REMARKS

Claims 1-24 and 27-29 are pending in the application. Claims 25 and 26 were previously canceled. No claims are canceled herein. Thus, claims 1-24 and 27-29 remain under consideration.

Claim 1 is amended herein. No new matter is added by these amendments. Support for the amendments is found throughout the as-filed specification and original claims.

I. 35 U.S.C. § 102(b) Su.

Claims 1-3, 6, 7, 12, 13, 17, 20 and 21 are rejected under 35 U.S.C. § 102(b) as allegedly anticipated by an English translation of CN 8910511.4 (Su). Su, however, fails to disclose that a sperm type is separated from a sperm population through an ion-permeable membrane. For at least this reason, Su does not anticipate the rejected claims.

In making the rejection of claim 1 based on Su, the Examiner states that the "semi-permeable bag" of Su is equivalent to the ion-permeable barrier of the presently rejected claims because Su's semi-permeable bag must be permeable to ions. Assuming, *arguendo*, that the Examiner is correct in this regard, Su still does not teach that a sperm type is separated from a sperm population *through* an ion-permeable barrier (emphasis added), which is required by all of the rejected claims.

In other words, even if Su does teach an ion-permeable barrier, it fails to disclose separating a sperm type by moving through that barrier when an electric field is applied. Figures 1 and 2 of Su illustrate embodiments lacking an ion-permeable barrier through which a sperm would move.

To make the distinction between Su and claim 1 even more clear, claim 1 has been amended to recite that a "sperm type moves through an ion-permeable barrier and is separated from a sperm population through the ion-permeable barrier." Su fails to show movement of sperm through an ion-permeable barrier. The Applicants, therefore, respectfully request that the 102(b) rejection of claim 1 be withdrawn. Because claims 2-3, 6, 7, 12, 13, 17, 20, and 21 all ultimately depend from claim 1 and include all of the elements of claim1, Su also fails to

anticipate these claims and the Applicants request that the rejection of these claims be withdrawn as well.

II. 35 U.S.C. § 103(a) Moore in view of Speicher.

Claims 1, 5-10, 12, 13, and 17-19 are rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Moore et al., J. Reprod. Fert. 44, 329-332 (1975) (Moore) and Speicher et al., U.S. Pat. No. 6,638,408 (Speicher). The Applicants respectfully traverse this rejection. Since claims 5-10, 12, 13 and 17-19 all ultimately depend from claim 1, these claims are also non-obvious over the cited references.

In regard to claim 1, the Examiner alleges that Moore discloses a process of separating a sperm type from a sperm population by electrophoresis using a pH-gradient. The Examiner acknowledges that Moore fails to disclose use of an ion-permeable barrier. The Examiner further alleges that Speicher discloses a separation chamber partitioned by ion-permeable membranes. The Examiner acknowledges that Speicher fails to disclose separation of a sperm type from a sperm population. The Examiner, however, alleges that it would have been obvious to one skilled in the art to use the isoelectric focusing device of Speicher to practice the method of Moore with predictable results. The Applicants respectfully disagree for the following reasons.

The membranes described in Speicher have pores too small, less than 0.5 μm, to allow sperm to pass through. *See* Speicher, col. 5:55-65. Although the Examiner states that Speicher describes that the samples may be cell or tissues samples, *see* June 11, 2010 Office Action page 7, line 17, further review of Speicher shows that the only "cell samples" used were cell free extracts of cells or tissues. *See, e.g.*, Example 1, col. 11:46-47; Example 6, col. 14:45-47, and Example 8, col. 16:29-31. Thus, the combination of Moore and Speicher would be inoperable to separate a sperm type from a sperm population through an ion-permeable barrier.

"An inference that a combination would not have been obvious is especially strong where the prior art's teachings undermine the very reason being proffered as to why a person of ordinary skill would have combined the known elements." DePuy Spine Inc. v. Medtronic

Sofamor Danek, Inc. 567 F.3d 1314, 1326 (Fed Cir. 2009). *See also* Examination Guidelines Update: Developments in the Obviousness Inquiry After KSR v. Teleflex, Fed. Reg. 53,643, 53,649 (Sept 1, 2010). Here the reason proffered for the combination of Moore and Speicher is to separate sperm, which cannot be achieved with the combination because of the membrane characteristics of Speicher. In other words, any method disclosed by Moore and Speicher in combination would not have worked for the intended purpose of separating sperm.

Furthermore, Moore discloses that the sperm cells separated using its method were "immotile." *See* Moore, page 330, line 16. As noted in the Applicants' response to the September 10, 2009 Office Action, the claimed processes produce sperm which are "substantially unchanged." For example, sperm separated by the claimed processes maintain their fertilizing potential and other properties such as motility. Thus, assuming, *arguendo*, that the combined references are operable, which they are not, the results of the claimed processes provide surprising and unexpected results over the cited references.

For these reasons, Applicants respectfully request the withdrawal of the rejection of claim 1. Since claims 5-10, 12, 13 and 17-19 all ultimately depend from claim 1 and include all the elements of claim 1, these claims are also non-obvious over the cited references, and the Applicants request withdrawal of the rejection of these claims as well.

III. 35 U.S.C. § 103(a) Moore in view of Speicher in further view of Barbour.

Claim 11 is rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Moore, as modified by Speicher, and in further view of Barbour et al., U.S. Patent No. 5,436,000 (Barbour). Claim 11 ultimately depends from claim 1. The Applicants, therefore, respectfully traverse this rejection for the same reasons described above regarding the rejection of claim 1 based on Moore as modified by Speicher.

Specifically, as described above, any method disclosed or suggested by Moore and Speicher in combination is inoperable for separating a sperm type. The Examiner incorrectly states that Speicher describes a pore size of as small as 0.5 microns. As noted above, 0.5

microns is the upper pore size limit described in Speicher, and this pore size is too small to allow sperm to pass. *See* Speicher, col. 5:55-65.

Moreover, claim 11 provides unexpected results in view of Moore as modified by Speicher. Moore and Speicher describe immotile sperm and no separation of sperm, respectively, so their combination fails to disclose or suggest the results achieved using the process of claim 11. Barbour does not overcome the deficiency of the Moore and Speicher combination in this regard. Barbour is cited as disclosing polycarbonate membrane as an ion-permeable barrier. The cited disclosure of Barbour is unrelated, however, with whether sperm separated through a membrane by a method resulting from the combination of Moore as modified by Speicher would be substantially unchanged. Thus, Barbour fails to disclose or suggest the results achieved with the process of claim 11, even when Barbour is read in combination with Moore and Speicher. The Applicants, therefore, respectfully request withdrawal of this rejection.

IV. 35 U.S.C. § 103(a) Moore in view of Speicher in further view of Moore II.

Claims 2, 3 and 14 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Moore, as modified by Speicher, and in further view of Moore, Int. J. Andro. 2: 449-452 (1979) (Moore II). Claims 2, 3 and 14 ultimately depend from claim 1. The Applicants, therefore, respectfully traverse this rejection for the same reasons described above regarding the rejection of claim 1 based on Moore as modified by Speicher.

Specifically, as described above, any method disclosed or suggested by Moore and Speicher in combination is inoperable for separating a sperm type. Moreover, claims 2, 3 and 14 each provide surprising and unexpected results in view of Moore as modified by Speicher. Moore and Speicher describe immotile sperm and no separation of sperm, respectively, so their combination fails to disclose or suggest the processes of claims 2, 3 and 14. Moore II fails to overcome the deficiency of the Moore and Speicher combination. Moore II is cited as teaching that spermatozoa from some "apparently infertile men have an isoelectric point consistently higher than fertile men." The cited disclosure of Moore II is unrelated, however, with whether

sperm separated through a membrane by a method resulting from the combination of Moore as modified by Speicher would be substantially unchanged. Thus, Moore II fails to disclose or suggest make the results achieved with the processes of claims 2, 3, or 14, even when Moore II is read in combination with Moore and Speicher. The Applicants, therefore, respectfully request withdrawal of this rejection.

V. 35 U.S.C. § 103(a) Moore in view of Speicher in further view of Jaspers, Raptis and Burke Jr.

Claims 14 and 15 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Moore, as modified by Speicher, and in further view of Jaspers et al., App. Evn. Micro. 3176-3181 (1997) (Jaspers), Raptis, U.S. Pat. No. 6,001,617 (Raptis), and Burke Jr. et al., U.S. Pat. App. Pub. No. 2002/0119218 (Burke Jr.). Claims 14 and 15 ultimately depend from claim 1. The Applicants respectfully traverse these rejections for the same reasons described above regarding the rejection of claim 1 based on Moore as modified by Speicher.

Specifically, as described above, the combination of Moore and Speicher is inoperable for separating a sperm type. Moreover, claims 14 and 15 each provide surprising and unexpected results in view of Moore as modified by Speicher. Moore and Speicher describe immotile sperm and no separation of sperm, respectively, so their combination fails to disclose or suggest the results achieved using the processes of claims 14 and 15. Jaspers, Raptis and Burke Jr., are cited for their described voltage gradients. The cited disclosures of these references are unrelated, however, with whether sperm separated through a membrane by a method resulting from the combination of Moore as modified by Speicher would be substantially unchanged. Thus, Jaspers, Raptis and Burke Jr. fails to disclose or suggest the results achieved with the processes of claim 14 and 15, even when the cited references are read in combination with Moore and Speicher. The Applicants, therefore, respectfully request withdrawal of this rejection.

VI. 35 U.S.C. § 103(a) Engelmann in view of Weber.

Claims 1-10, 12-23, 28 and 29 are rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Engelmann et al., Gamete Res. 19, 151-159 (1988) (Engelmann), which was cited in the September 10, 2009 Office Action, in view of Weber, U.S. Patent No. 7,399,394 (Weber). In regard to the rejection of claim 1, the Examiner uses similar reasoning to the reasoning used to reject claim 1 based on Engelmann and Rylatt in the September 10, 2009 Office Action. See Office Action dated September 10, 2009, pages 3-4.

For example, in making the current rejection based on Engelmann and Weber, the Examiner alleges that Engelmann teaches a method of sperm separation based on elecrophoretic mobility and that Weber discloses a method for separating charged substances with an ion-permeable membrane. Rylatt was cited previously for its teaching of an ion-permeable membrane and was combined with Engelmann.

In their response to the September 10, 2009 Office Action, the Applicants pointed out that Engelmann discloses or suggests that the motility of sperm separated by free flow electrophoresis is "greatly reduced." See Engelmann, page 156. The Applicants further argued that one skilled in the art would expect even more damage to separated sperm if an ion-permeable membrane was used to separate the sperm. See Amendment and Response to Office Action of September 10, 2009 page 8. For these reasons, the Applicants explained that the results achieved using the process of claim 1 was surprising and unexpected since it showed less damage to separated sperm, not more, as would be expected by using the free-flow electrophoresis of Engelmann with the ion-permeable membrane of Rylatt. The current rejection is essentially the same; and, for these same reasons, one skilled in the art would not expect a separated sperm type having less damage than Engelmann if combined with Weber. Instead, one skilled in the art would expect a sperm type with even less motility than the "greatly reduced" motility described in Engelmann. Because the process of claim 1 results in surprisingly superior results over the cited references, it is non-obvious over the cited references. Since claims 2-10, 12-23, 28 and 29 all ultimately depend from claim 1 and include all of the elements of claim 1,

the Applicants respectfully request withdrawal of the rejection as to claim 1 and these dependent claims.

VII. 35 U.S.C. § 103(a) Engelmann in view of Weber in further view of Barbour.

Claim 11 is rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable over Engelmann as modified by Weber and in further view of Barbour. Claim 11 ultimately depends from claim 1. As explained above, the results of claim 1, and therefore of claim 11, are surprising and unexpected in light of Engelmann as modified by Weber. Barbour is cited for its disclosure of polycarbonate membrane materials. The disclosure of Barbour does not make the results of claim 11 predictable in light of the Engelmann and Weber combination. The Applicants, therefore, respectfully request withdrawal of this rejection.

VIII. 35 U.S.C. § 103(a) Su in view of Christensen.

Claims 18 and 19 are rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Su in view of Christensen et al., U.S. Patent No. 7,070,917(Christensen). As described above, Su does not teach or suggest that a sperm type moves through an ion-permeable barrier and is separated from a sperm population through the ion-permeable barrier. Christensen is cited for a device to count bovine sperm. Thus, the combination of Su and Christensen fails to disclose or suggest that a sperm type moves through an ion-permeable barrier and is separated from a sperm population through the ion-permeable barrier, which is required by claims 18 and 19. The Applicants, therefore, respectfully request withdrawal of this rejection.

IX. 35 U.S.C. § 103(a) Su in view of Kricka.

Claim 27 is rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable over Su in view of Kricka et al., U.S. Patent No. 5,427,946 (Kricka). As described above, Su fails to disclose or suggest that a sperm type moves through an ion-permeable barrier and is separated from a sperm population through the ion-permeable barrier. Kricka is cited for fertilization of an ovum with viable sperm. Thus, the combination of Su and Kricka fails to disclose or suggest

that a sperm type moves through an ion-permeable barrier and is separated from a sperm population through the ion-permeable barrier, which is required by claim 27. The Applicants, therefore, respectfully request withdrawal of this rejection.

X. 35 U.S.C. § 103(a) Moore in view of Speicher in further view of Kricka.

Claim 27 is rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable over Moore as modified by Speicher and in further view of Kricka. As described above, Moore and Speicher are inoperable for separating a sperm type. Moreover, their combination fails to disclose or suggest the unexpected results achieved using the method of claim 27. Claim 27 depends from claim 1, which, as described above, provides unexpected results over Moore in view of Speicher. Kricka is cited for fertilization of an ovum with viable sperm. The cited disclosure of Kricka is unrelated, however, with whether sperm separated through a membrane by a method resulting from the combination of Moore as modified by Speicher would be substantially unchanged. Thus, Kricka fails to disclose or suggest the results achieved with the method of claim 27, even when Kricka is read in combination with Moore and Speicher. The Applicants, therefore, respectfully request withdrawal of this rejection.

XI. 35 U.S.C. § 112, first paragraph.

Claim 2 is rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement for separating a sperm based on genetic makeup or morphological normality. The Examiner, however, concedes that claim 2 is enabled for separating a sperm based on motility, robustness, gender, and fertilizing potential. The Applicants respectfully traverse this rejection. The Examiner is improperly reading limitations into the claim that do not exist and then alleging that these non-existent limitations are not enabled.

Claim 2 does not recite or require that a sperm is separated "based on" genetic makeup or morphological normality. Claim 2 recites a process for separating a sperm type from a sperm population in a sperm sample by electrophoresis comprising subjecting the sperm population to an electric potential such that a sperm type moves through an ion-permeable barrier and is

separated from a sperm population through the ion-permeable barrier wherein the separated sperm type has a desired characteristic. The desired characteristic can be genetic makeup or morphological normality.

The recitation of "desired characteristic" does not require that the selection was "based on" that characteristic. The separated sperm population does not need to be separated "based on" genetic make-up or morphological normality for the separated sperm to have a desired genetic make-up or morphological normality. When the rejected claims are read in this light, e.g., without the improper limitation, the data provided in the application show that sperm cells with morphological normality and a desired genetic make-up were separated using the claimed process.

For example, paragraph 111 of the specification states that a statistically significant higher proportion of the spermatozoa with normal morphology were found in the separated fraction as compared to the excluded fraction ($p<0.001$). Furthermore, paragraph 113 of the specification describes that the separated populations expressed significantly lower levels of detectable DNA damage ($p<0.001$). Thus, the claimed process is enabled for separating a sperm type from a sperm population, wherein the sperm type has a desired characteristic including higher normal morphology and lower DNA damage (e.g., desired genetic make-up).

The Examiner alleges that the data showing that the separated sperm type with a desired characteristic of normal morphology lack significance. *See* June 11, 2010 Office Action page 23. The data from paragraph 111, discussed above, indicates a p-value of < 0.001, a very high of statistical significance, regarding morphology in the separated sperm type. Thus, the data are statistically significant and the claims are enabled. The Applicants respectfully request withdrawal of the rejection of claim 2.

Claim 4 is rejected under 35 § U.S.C. 112, first paragraph, for lacking enablement for separating sperm based on poor morphology, high levels of DNA damage, and high levels of reactive oxygen species generation. The Examiner concedes that the method for separating sperm based on poor motility is enabled. In regard to separating sperm "based on" poor morphology, high levels of DNA damage and high levels of reactive oxygen species generation,

the Applicants again respectfully assert that the "based on" limitation is being improperly read into the claim. Claim 4 does not require that separation is based on poor morphology, high levels of DNA damage or high levels of reactive oxygen species generation. Similar to the arguments provided above in relation to rejection of claim 2 under 35 U.S.C. § 112, first paragraph, the claims are enabled for separation of a sperm type that has these undesired characteristics.

The data in the application demonstrate separation of sperm types having undesired characteristics such as poor morphology and high levels of DNA damage. For example, in paragraph 111, the excluded fraction of sperm (e.g., a sperm type) had significantly higher levels of poor morphology ($p<0.001$) and in paragraph 113, the excluded fraction had significantly higher levels of DNA damage ($p<0.001$). Thus, in both cases, a sperm type was separated wherein the sperm type had either an undesirable characteristic of poor morphology or high DNA damage.

In regard to the enablement of separating a sperm type having high levels of reactive oxygen species generation, the as-filed specification describes separation of a sperm type having high levels of oxygen species generation by subjecting a sperm population to an electric potential such that a sperm type moves through an ion-permeable barrier. Furthermore, Aitken et al., *Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence,* J Cell Physiol, 151:466–477 (1992) (attached as Exhibit A), is a pre-effective-filing-date reference describing a chemiluminescence assay for detecting reactive oxygen species generation from sperm. Aitken, therefore, demonstrates that one skilled in the art at the time of filing would have known that separated sperm could be assayed for high levels of oxygen species generation as described in the application. At the time of filing, one skilled in the art would have therefore known how to separate sperm and how to determine that the separated sperm had high levels of reactive oxygen species generation using the claimed method, without undue experimentation, based on the as-filed specification and the knowledge of one skilled in the art. Therefore, claim 4 is enabled for separating a sperm type from a sperm population in a sperm sample by electrophoresis

comprising subjecting the sperm population to an electric potential such that a sperm type moves through an ion-permeable barrier, and is separated from a sperm population through the ion-permeable barrier wherein the separated sperm type has the undesired characteristic of high levels of oxygen species generation.

For further evidence that claim 4 is enabled for separation of a sperm type having high levels of oxygen species generation, the Applicants provide Ainsworth et al., *Development of a novel electrophoretic system for the isolation of human spermatozoa*, Hum. Reprod., 20:8, 2216-2270 (2005) (Ainsworth) (attached as Exhibit B), which is a post-effective-filing-date reference. Although enablement is determined as of the effective filing date, post-filing evidence can be used to prove that the disclosure was in fact enabled when filed. *In re Brana*, 51 F.3d 1560, 1567 (Fed. Cir. 1995). Ainsworth demonstrates that one skilled in the art at the time of filing could have used the described methods, without undue experimentation, to separate a sperm type having high levels of oxygen species generation and could have determined that the sperm type possessed this undesired characteristic using the known assay of Aitken. Specifically, Figure 3 (shown below) from Ainsworth demonstrates that prior to administration of Zymosan the chemiluminescense of sperm separated by subjecting a sperm population to an electric potential such that a sperm type moved through an ion-permeable barrier was lower than the chemiluminescense of the residual sperm type that did not move through the barrier.

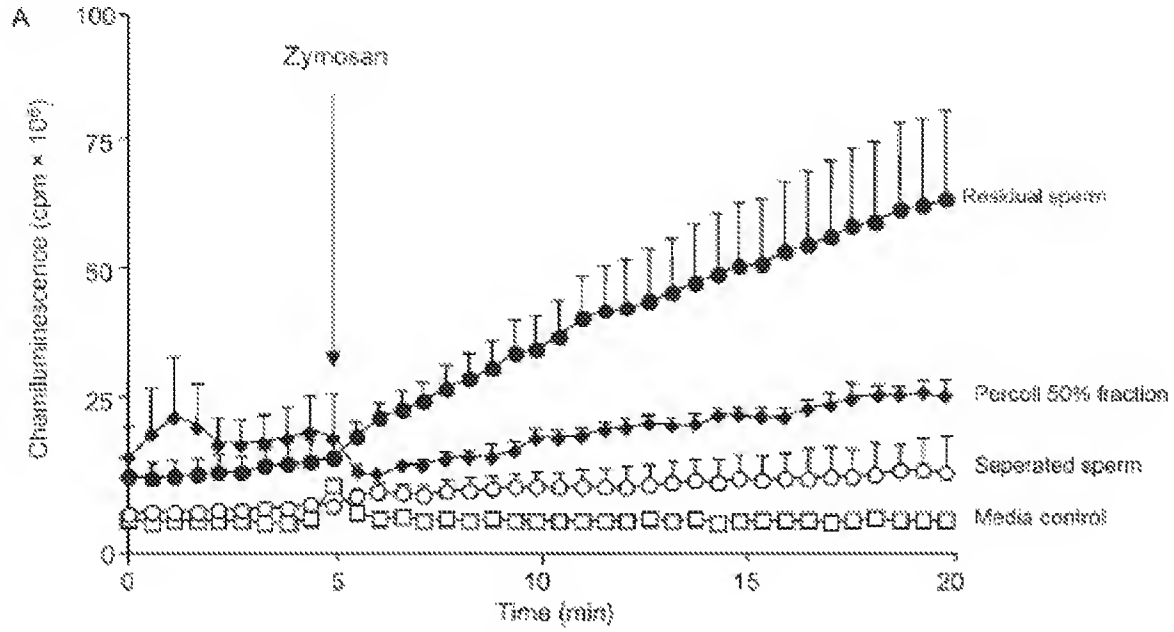

Figure 3 from Ainsworth et al., Hum. Reprod. (2005).

The chemiluminescense assay used in Ainsworth was described in Aitken and indicates reactive oxygen species generation from human spermatozoa. Therefore, Ainsworth's Figure 3 demonstrates that a residual sperm type was separated from a sperm population and that the sperm type had high levels of reactive oxygen species generation.

Because the as-filed and the post-effective-filing-date data demonstrate separation of sperm types having undesired characteristics of poor morphology, high levels of DNA damage and high levels of reactive oxygen species generation, and because the Examiner has conceded enablement for poor motility, the Applicants respectfully request withdrawal of the rejection of claim 4.

XII. Conclusions.

In view of the amendments and arguments herein, Applicants respectfully request allowance of all claims.

It is believed that all issues raised by the Examiner have been addressed. However, the absence of a reply to a specific rejection, issue, or comment does not signify agreement with or concession of that rejection, issue, or comment. In addition, because the arguments made above may not be exhaustive, there may be reasons for patentability of any or all pending claims (or other claims) that have not been expressed. Finally, the amendment of any claim does not necessarily signify concession of unpatentability of the claim prior to its amendment.

Fees in the amount of $245.00 for a two-month extension of time for a small entity are being paid concurrently herewith on the Electronic Filing System by way of Electronic Funds Transfer authorization. Please apply any other charges or credits to Deposit Account 50-5226.

Respectfully submitted,

Date: October 27, 2010

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EXHIBIT A

JOURNAL OF CELLULAR PHYSIOLOGY 151:466–477 (1992)

Reactive Oxygen Species and Human Spermatozoa: Analysis of the Cellular Mechanisms Involved in Luminol- and Lucigenin-Dependent Chemiluminescence

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We have shown that human spermatozoa generate and release reactive oxygen species that can be detected by chemiluminescence techniques. Analysis of the cellular mechanisms responsible for this activity suggests that the probe, luminol, undergoes an intracellular dioxygenation reaction mediated by hydrogen peroxide and a sperm peroxidase located within the acrosome. Support for this model included the following observations: (1) the luminol-dependent signal could be suppressed with peroxidase inhibitors, phenylhydrazine and sodium azide; (2) this suppression could be reversed by the addition of an azide-insensitive peroxidase, horse radish peroxidase (HRP); (3) inhibition of intracellular superoxide dismutase (SOD) with potassium cyanide (KCN) suppressed the luminol signal; (4) peroxidase activity could be detected in purified populations of human spermatozoa with 3,3′,5,5′ tetramethylbenzidine (TMB); (5) this peroxidase was active at the pH prevailing within the acrosomal vesicle; and (6) peroxidase activity and luminol-dependent chemiluminescence were minimal in spermatozoa exhibiting a congenital absence of acrosomes. Human spermatozoa could also generate lucigenin-dependent chemiluminescent signals that could neither be suppressed with peroxidase inhibitors nor enhanced by the addition of peroxidase. However, these signals could be enhanced by suppression of intracellular SOD with KCN or inhibited by exogenous SOD, suggesting that lucigenin was responding to superoxide anion released into the extracellular space. The ability of chemiluminescent techniques to detect and discriminate the production of superoxide and hydrogen peroxide by spermatozoa should facilitate the further analysis of reactive oxygen species as mediators of normal and abnormal human sperm function.

An intriguing, highly specialized feature of the human spermatozoon is the capacity of this cell type to generate reactive oxygen species, including superoxide anion and hydrogen peroxide (Aitken and Clarkson, 1987; Alvarez et al., 1987). The mechanisms responsible for superoxide generation in human spermatozoa bear a superficial resemblance to the superoxide-generating system of phagocytes in terms of their dependence upon NADPH as a source of electrons and their responsiveness to activators of protein kinase C (Aitken, 1990). However, clear differences in the superoxide-generating systems possessed by these cell types also exist in that spermatozoa are completely unresponsive to classical activators of the leukocyte NADPH oxidase, such as formylmethionyl-leucyl-phenylalanine (Krausz et al., 1992).

The generation of reactive oxygen species by human speratozoa has been implicated in the control of normal sperm function (Aitken et al., 1989a) and in the aetiology of male infertility associated with a peroxidation-induced loss of plasma membrane function (Jones et al., 1979; Mennella and Jones, 1980; Aitken and Clarkson, 1987; Alvarez et al., 1987; Aitken, 1989; Aitken et al., 1989a,b, 1991; Alvarez and Storey, 1989; Jeulin et al., 1989). In view of the clinical and functional significance of reactive oxygen species generation by human spermatozoa, the development of sensitive techniques to monitor this activity is of some importance.

Ferricytochrome *c* reduction has been frequently used for measuring the extracellular concentration of superoxide anion in phagocytes and this technique has been successfully applied to human spermatozoa in the studies of Alvarez et al. (1987). This procedure has advantages in terms of its specificity for superoxide anion but lacks the sensitivity needed to explore the generation of this radical in cases of severe oligozoospermia and does not allow real time analysis of response kinetics. Furthermore, this procedure cannot generate information on the intracellular generation of reactive oxygen species. As a result of these factors, attention has

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focused on the use of chemiluminescent probes, which have been extensively used to investigate the production of reactive oxygen species by leucocytes (Allen and Loose, 1976; Hallett and Campbell, 1983; Wymann et al., 1987a,b; Vilim and Wilhelm, 1989) and endothelial cells (Sundqvist, 1991) to monitor this activity in human spermatozoa (Aitken and Clarkson, 1987). Preliminary clinical studies have already indicated that the luminol-dependent chemiluminescent signals generated by human spermatozoa are of prognostic value (Aitken et al., 1989b, 1991). However, the interpretation of these data has been confounded by our poor understanding of the cellular mechanisms by which such signals are generated.

A variety of oxygen radicals, including superoxide anion and the hydroxyl radical, as well as nonradical oxidants such as hydrogen peroxide, are potential mediators of luminol-dependent chemiluminescence (Allen, 1982), the precise nature of the oxidizing species depending on the cell type under consideration. Hence, the chemiluminescence response given by neutrophils has been well characterized and shown to involve an azide-sensitive intracellular peroxidase (myeloperoxidase), which catalyses the dioxygenation of luminol by intracellular hydrogen peroxide (De Chatelet et al., 1982; Hallett and Campbell, 1983; Dahlgren and Stendahl, 1983). In contrast, the luminol-dependent chemiluminescence exhibited by alveolar macrophages appears to involve the mediation of superoxide in the complete, or virtual, absence of peroxidase activity (Welch et al., 1980; Vilim et al., 1984; Vilim and Wilhelm, 1989). Yet again, the luminol-dependent chemiluminescence given by peritoneal macrophages is thought to depend upon hydroxyl radicals formed during the metabolism of arachidonic acid (Lim et al., 1983; Vilim and Wilhelm, 1989). The purpose of this study was to establish the mechanisms by which human spermatozoa generate luminol-dependent chemiluminescence and to use this information to refine the protocols used for monitoring reactive oxygen species generation by these cells.

MATERIALS AND METHODS

Materials

The semen samples were obtained from a panel of unselected donors exhibiting semen profiles that were normal according to the criteria laid down by the World Health Organization (WHO, 1987), i.e., sperm concentration $> 20 \times 10^6$/ml; sperm morphology > 50% normal; sperm motility > 50% motile. In addition all of the donors had been screened for hepatitis and sexually transmitted diseases, including HIV.

Sperm preparation

The samples were produced by masturbation and allowed to liquify at 37°C for at least 30 min. The spermatozoa were subsequently separated from the seminal plasma on discontinuous Percoll® gradients as described by Aitken and Clarkson (1988) except that a simplified two-step gradient was created in a 15 ml sterile centrifuge tube by overlaying 3 ml of 80% or 100% Percoll® with 3 ml of 40% Percoll®. Isotonic Percoll® was created by supplementing 10 ml of 10x concentrated medium 199 (Flow Laboratories, Irvine, Scotland) with 300 mg BSA, 3 mg sodium pyruvate, and 0.37 ml of a sodium lactate syrup and adding 90 ml of Percoll® (Pharmacia, Uppsala, Sweden). This preparation was designated 100% Percoll® (Lessley and Garner, 1983) and was subsequently diluted with HEPES-buffered medium BWW (Biggers et al., 1971). After a 20-min period of centrifugation at 500 g, the spermatozoa at the base of each Percoll® fraction were collected separately, washed with a 5 ml volume of medium BWW, and finally resuspended in BWW at a concentration of 20×10^6/ml.

The absence of leucocyte contamination in the high density Percoll® fractions was confirmed using a monoclonal antibody against the common leucocyte antigen (Scottish Antibodies Production Limited, Carluke, Scotland; S064-201, clone 2B11 + PD 7/26 and S149-201, clone 17.21) in the alkaline phosphatase/anti-alkaline phosphatase protocol described by Aitken and West (1990).

Chemiluminescence

Reactive oxygen species production was measured by chemiluminescence as described by Aitken and Clarkson (1987). The chemiluminescent signals were recorded on a Berthold, L139 500T luminometer in the integration mode using a capture time of 10 sec. The probe employed was luminol (amino-2,3-dihydro-1,4-phthalazinedione), which undergoes a dioxygenation reaction in the presence of reactive oxygen species to yield a cyclic peroxide that disintegrates to yield the electronically excited aminophthalate anion. At neutral pH, luminol is undissociated and exhibits a tendency toward hydrophobic binding (Allen, 1982). As a consequence of this relative membrane permeability, luminol has been used extensively to monitor the generation of reactive oxygen species from intracellular sites, such the phagolysosome of polymorphonuclear leucocytes (Allen, 1982).

The second probe employed was lucigenin, the chemiluminescence of which appears to involve the superoxide-mediated generation of excited N-methylacridone, via a stepwise electron reduction and oxygenation pathway involving a radical intermediate (Allen, 1982). At neutral pH, lucigenin is present as a membrane impermeant divalent cation and has been applied clinically to examine the release of reactive oxygen species into the extracellular space from activated leucocytes.

Luminol and lucigenin were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, Cat Nos. A8511 and M8010) and stored in the dark at 4°C in dimethylsulfoxide. The working concentrations for both chemiluminescent probes was 250 μM and the chemiluminescent signals were recorded in 400 μl sperm suspensions containing 10×10^6 spermatozoa/ml.

Horseradish peroxidase (HRP; Sigma, type VI, Cat. No. P8375) at a final concentration of 12 purpurogallin units/ml was used to enhance the chemiluminescent signal in certain experiments. The influence of a number of potential inhibitors of chemiluminescent reactions was also examined, including superoxide dismutase (33 U), which catalyses the dismutation of

superoxide to hydrogen peroxide (Calbiochem, San Diego, CA, Cat. No. 574594), catalase (23 kU), which catalyses the breakdown of hydrogen peroxide to oxygen and water (Sigma, Cat. No. C-10, 2,600 Units/mg), azide (10.0 μM), primarily as an inhibitor of endogenous peroxidase activity (Sigma, Cat. No. 2002) and 5 mM potassium cyanide (BDH Chemicals, Poole, U.K.), as an inhibitor of intracellular superoxide dismutase activity.

Hydrogen peroxide

Hydrogen peroxide levels were monitored in a fluorometric assay employing homovanillic acid (Sigma, Cat No. H1252) as the substrate. The reaction mixture contained 500 μl spermatozoa at a concentration of 20×10^6/ml, 15 μl of a 10 mM solution of homovanillic acid and 6 μl (3.7 purpurogallin Units) HRP; the excitation and emission wavelengths employed were 315 and 425 nm, respectively.

Peroxidase activity

Peroxidase activity was measured in suspensions of human spermatozoa using the tetramethylbenzidine (TMB; Sigma, Cat. No. T5525) substrate described by Suzuki et al. (1983). For this assay, 100×10^6 human spermatozoa recovered from the base of the high density Percoll® fraction were extracted for 1 h at 37°C in an 0.5 M tris acetate buffer, pH 5.0, containing 0.5% Triton ×100. The cells were subsequently pelleted by centrifugation at 1,470 g for 5 min and the supernatants assayed for peroxidase activity. Absorbance was measured at 650 nm following the addition of 1 ml of 1.2% hydrogen peroxide in tris-acetate buffer to a mixture containing 0.1 ml of the sperm extract and 0.1 ml of TMB (5 mg/ml). Inhibition of the peroxidase activity was achieved with phenylhydrazine and sodium azide, both purchased from Sigma (Cat. Nos. P6926 and S2002).

Data analysis

All experiments were replicated at least three times and, where indicated, the statistical significance of differences was assessed using nonparametric statistics (Wilcoxon signed rank test).

RESULTS

Luminol-dependent chemiluminescence

Human spermatozoa, purified on Percoll® gradients and free from contaminating leucocytes or precursor cells, were shown to generate a basal, steady-state, luminol-dependent chemiluminescence that could be readily suppressed by the addition of 10 μM sodium azide (Fig. 1a). The chemiluminescent responses given by human spermatozoa could be stimulated by the addition of 5 μM A23187 or 100 nM PMA (12 myristate, 13 acetate phorbol ester) with the latter generating responses of greater consistency and magnitude (see the ordinate axes of Figs.1b,d and 2c,e,f). The responses induced by these agonists were also shown to be susceptible to azide inhibition in time-dependent analyses (Fig. 1b,d). The statistical significance ($P < 0.01$) of these suppressive effects was confirmed in a cohort study by measuring the chemiluminescent signals generated by eight independent samples at a single time point of 3 min (Aitken and Clarkson, 1987; Fig. 2a,b). The suppressive effect of azide was immediate and could be observed whether this inhibitor was added before (compare Fig. 2c,d) at the same time as (Fig. 2a,b) or after (Fig. 1b,d) the delivery of stimulants such as A23187 or PMA.

The addition of exogenous HRP (12 purpurogallin Units was established as the most effective concentration in dose-dependent studies) elicited a significant increase in the basal, luminol-dependent signals given by human spermatozoa (Fig. 1c; $P < 0.01$ after 3 min, $n = 8$), although the extent of this elevation depended on the intensity of the basal signal. In sperm suspensions characterized by an elevated basal level of chemiluminescence, as might be encountered in the infertile population (Aitken and Clarkson, 1987), addition of HRP had an immediate stimulatory effect on the luminol-dependent signal (Fig. 1c). However, in sperm samples characterized by a low, steady-state level of chemiluminescence, as might be encountered in the normal fertile population (Aitken and Clarkson, 1987), addition of HRP had a less marked effect (Fig. 2e). In contrast, HRP consistently enhanced the chemiluminescent responses to A23187 ($P < 0.01$; $n = 8$; Fig. 2b) and PMA ($P < 0.01$; $n = 8$; Fig. 2a). In such instances the enhancing effect of peroxidase was immediate and was apparent whether the HRP was added before (compare ordinate axes of c and e, Fig. 2) at the same time as (Fig. 2a,b) or after (Fig. 3a,b) stimulation of the spermatozoa with A23187 or PMA.

Moreover, exogenous HRP could reverse the inhibitory effects of azide in both the steady-state situation (Fig. 1a) and in the presence of agonist (Figs. 2d,f, 3b). As a consequence, azide had no significant suppressive effect on the luminol-dependent chemiluminescent signals generated by human spermatozoa when added at the same time as (Fig. 2a,b) or after (Fig. 3c) these cells had been treated with peroxidase.

The enhancing effect of HRP on the basal chemiluminescent signal, as well as the response of the spermatozoa to A23187 or PMA, could be significantly suppressed ($P < 0.01$ after 3 min; $n = 7$) by the addition of either 33 U SOD or 23 kU of catalase, (Fig 3d,e), the latter consistently returning the chemiluminescent signal to baseline. Heat inactivation (80°C for 20 min) completely blocked the ability of these enzymes to suppress the peroxidase-enhanced chemiluminescent signal (Fig. 3e), indicating that the inhibition of HRP-mediated chemiluminescence was a specific event and not a consequence of nonspecific scavenging activity (Vilim and Wilhelm, 1989). SOD had a less dramatic effect on the luminol-dependent chemiluminescence in the absence of exogenous HRP (Fig. 3f) presumably because, in the absence of exogenous peroxidase, the biochemical reactions responsible for the chemiluminescence are intracellular and therefore inaccessible to membrane-impermeant extracellular enzymes. A similar argument applies to catalase, which in the absence of HRP exerts a statistically significant, though minimal effect on the luminol dependent signals generated generated by human spermatozoa in both the steady-state situation and following stimulation with A23187 (Aitken et al., 1989).

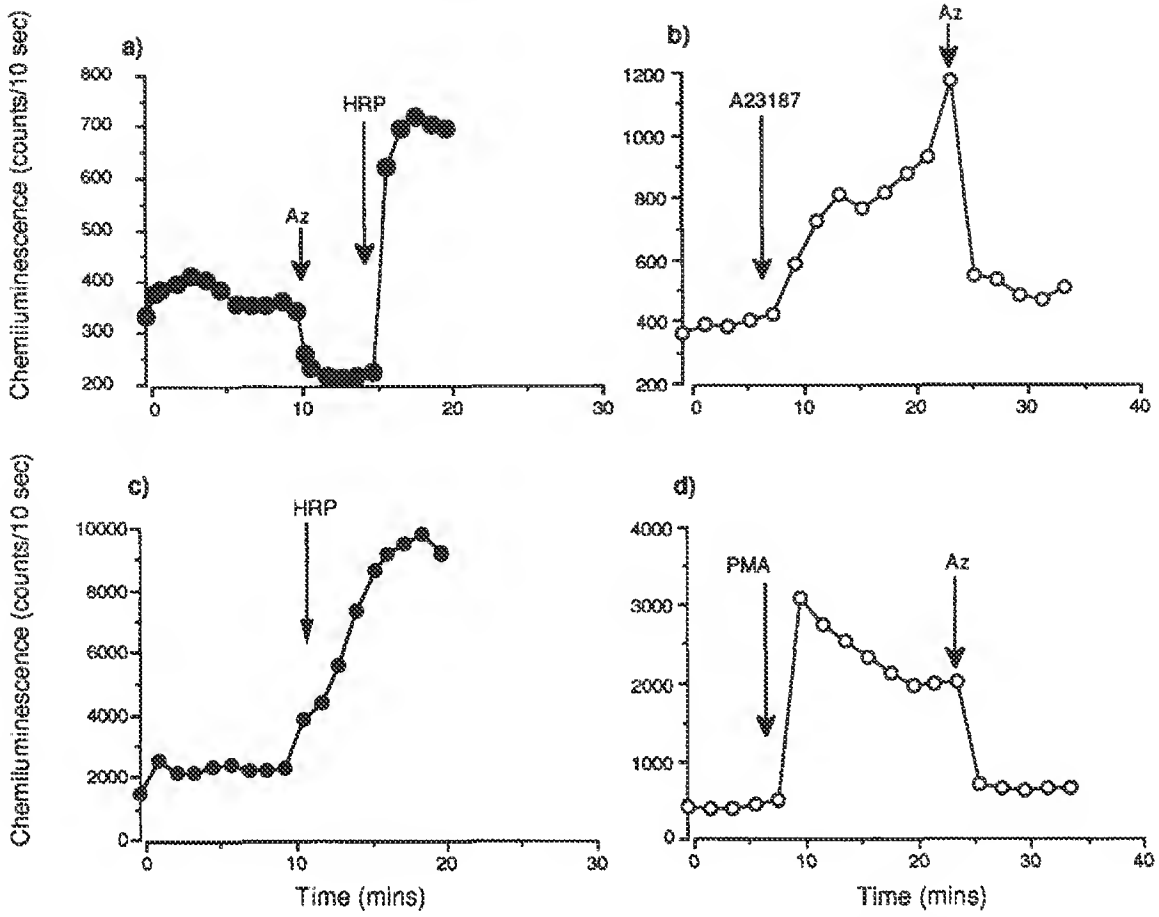

Fig. 1 Influence of azide on the luminol-dependent chemiluminescent signals generated by purified suspensions of human spermatozoa (a) in a steady state situation, (b) in response to 5 μM A23187, or (d) in response to 100 nM PMA. Note the ability of HRP, an azide insensitive peroxidase, to enhance the basal level of sperm chemiluminescence (c), and to reverse the inhibitory effects of azide (a).

The enhancing effect of HRP, together with the suppressive effects of catalase, suggested that the major reactive oxygen species released from human spermatozoa into the extracellular space is hydrogen peroxide. This conclusion was supported by the direct measurement of a catalase-inhibitable extracellular hydrogen peroxide signal in the presence of human spermatozoa, using a fluorometric assay based on the oxidation of homovanillic acid in the presence of HRP (Fig. 4). The amount of hydrogen peroxide generated was found to be significantly higher in the cell populations isolated on the low density portion of the Percoll® gradients, as previously demonstrated using chemiluminescence techniques (Aitken and Clarkson, 1988). The major difference between the fluorometric technique and chemiluminescence lay in the relative sensitivity of these methods. Using the HRP-supplemented chemiluminescence system employing 12 units of HRP and 250 μM luminol, hydrogen peroxide could be resolved at a concentration of pmoles/ml, in contrast to the nmoles/ml detectable with the homovanillic acid-based fluorescence system (Fig 4; Wymann et al., 1987).

If this hydrogen peroxide arises through the intracellular dismutation of superoxide anion, it follows that the treatment of these cells with a reagent such as KCN, which inhibits sperm SOD (Alvarez et al., 1987), should diminish the HRP-dependent luminol signal. Using sperm suspensions purified on Percoll® and free from contaminating leucocytes, 5 mM KCN was shown to have a suppressive effect on the chemiluminescent signal generated in response to PMA, in the presence of luminol and peroxidase (Fig. 3d). The residual response to PMA observed in the presence of KCN could be readily scavenged by extracellular catalase (Fig. 3d), suggesting that a significant proportion of the chemiluminescent signal recorded in the presence of this inhibitor, arose through the spontaneous dismutation of superoxide anion.

Sperm peroxidase

The sensitivity of sperm chemiluminescence to inhibition with azide was consistent with the notion that the luminol-dependent signals generated by human spermatozoa in the *absence* of HRP enhancement involved the mediation of an intracellular peroxidase. Using the peroxidase substrate TMB (Suzuki et al., 1983), peroxidase activity could be readily demonstrated in purified suspensions of human spermatozoa.

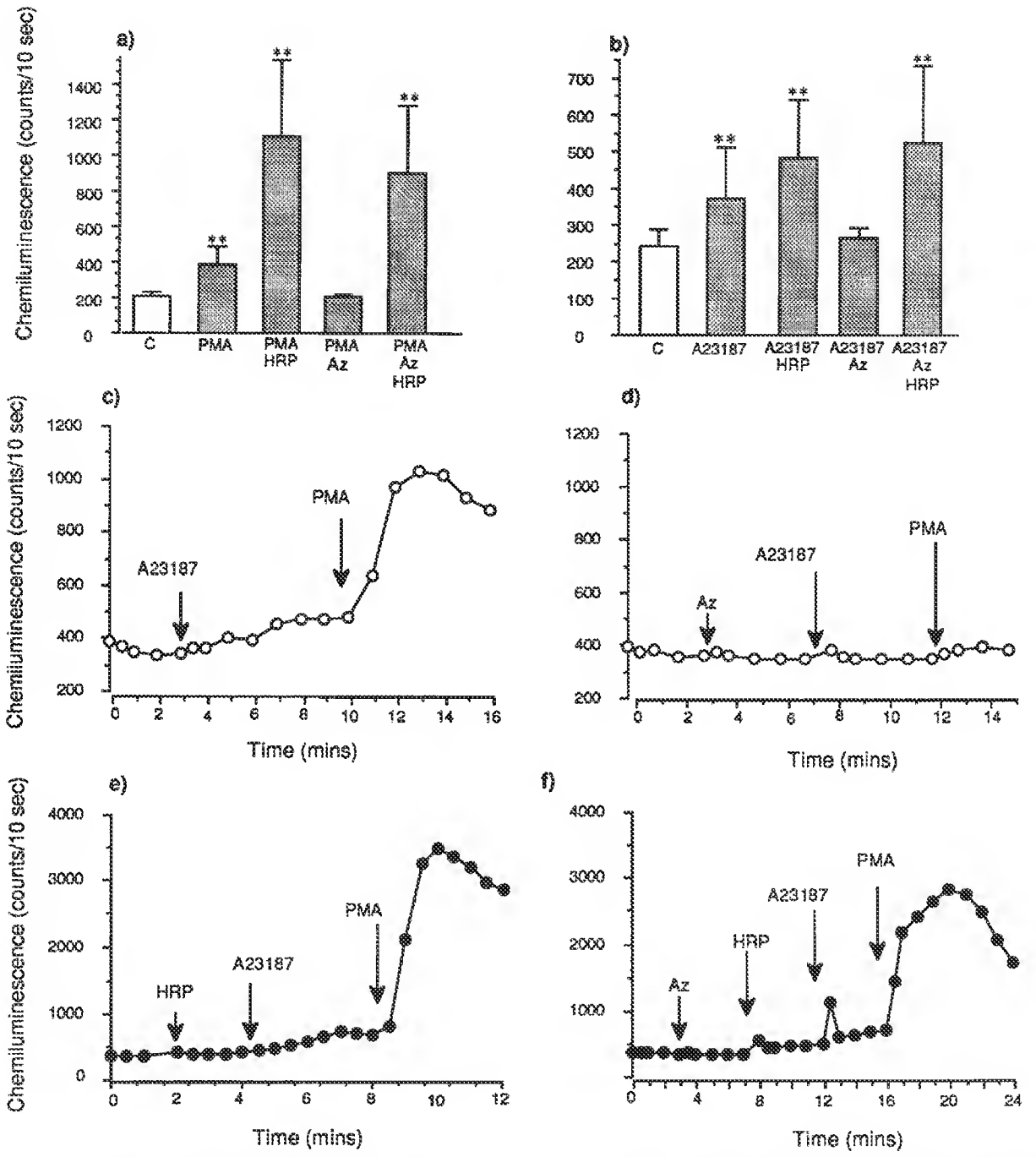

Fig. 2. Influence of azide (10 μM) and HRP (12 purpurogallin Units /ml) on the luminol-dependent chemiluminescent signals generated by human spermatozoa. (a and b) Cohort analyses involving eight independent sperm samples, sampled at a fixed time interval of 3 min after the simultaneous addition of HRP (12 purpurogallin Units /ml), azide (10 μM) and stimulant; median values, ** $P \leq 0.001$. (c–f) Longitudinal analyses of the responses of human spermatozoa to A23187 and PMA following pretreatment with (c) medium alone, (d) azide, (e) HRP, and (f) azide + HRP.

The peroxidase activity was strictly dependent on sperm concentration (Fig. 5a), was susceptible to peroxidase inhibitors such as phenylhydrazine and azide (Fig. 5b), and exhibited a pH optimum of 4.5–5.0 (data not shown).

In neutrophils there is good evidence to indicate that the luminol-dependent chemiluminescence is due to myeloperoxidase located in the acidic environment of the secretory granules (Vilim and Wilhelm, 1989). Since the sperm acrosome is held to be a secretory granule (Friend, 1977) with an acidic internal pH (Meizel and Deamer, 1978) and since the peroxidase activity detected in human spermatozoa with TMB was active at pH 5.0—the pH recorded in the acrosomal matrix by

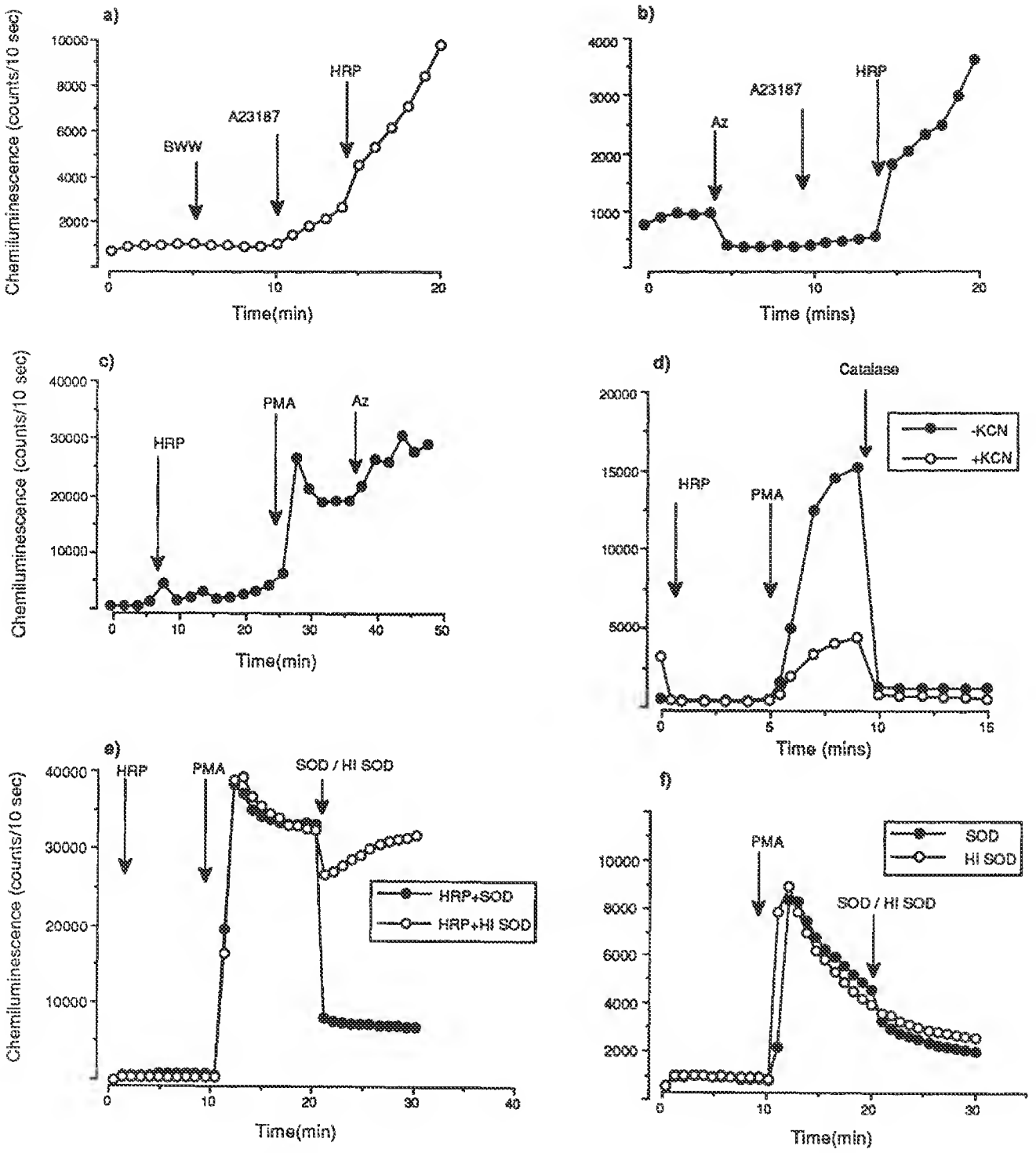

Fig. 3. Influence of azide (10 μM), HRP (12 purpurogallin Units /ml) and the scavenging enzymes SOD (33 U) and catalase (23 kU) on the chemiluminescent signals generated by human spermatozoa. (a and b) HRP enhancement of the response to 5 μM A23187 even after the signal had initially been suppressed by pretreatment with 10 μM azide (Az). (c) Azide had no significant effect on the chemiluminescent responses if the cells were pretreated with HRP. (d) Catalase (23 kU) completely suppressed the luminol-dependent signal generated by human spermatozoa in response to PMA. Catalase also suppressed the residual response to PMA after the spermatozoa had been exposed to KCN. (e) SOD (33 U) exerted an immediate suppressive effect on the HRP-enhanced response to PMA, although not to prestimulation levels. This inhibitory effect is removed by heat inactivation of the SOD (HI SOD). (f) SOD has less of an effect in the absence of extracellular HRP.

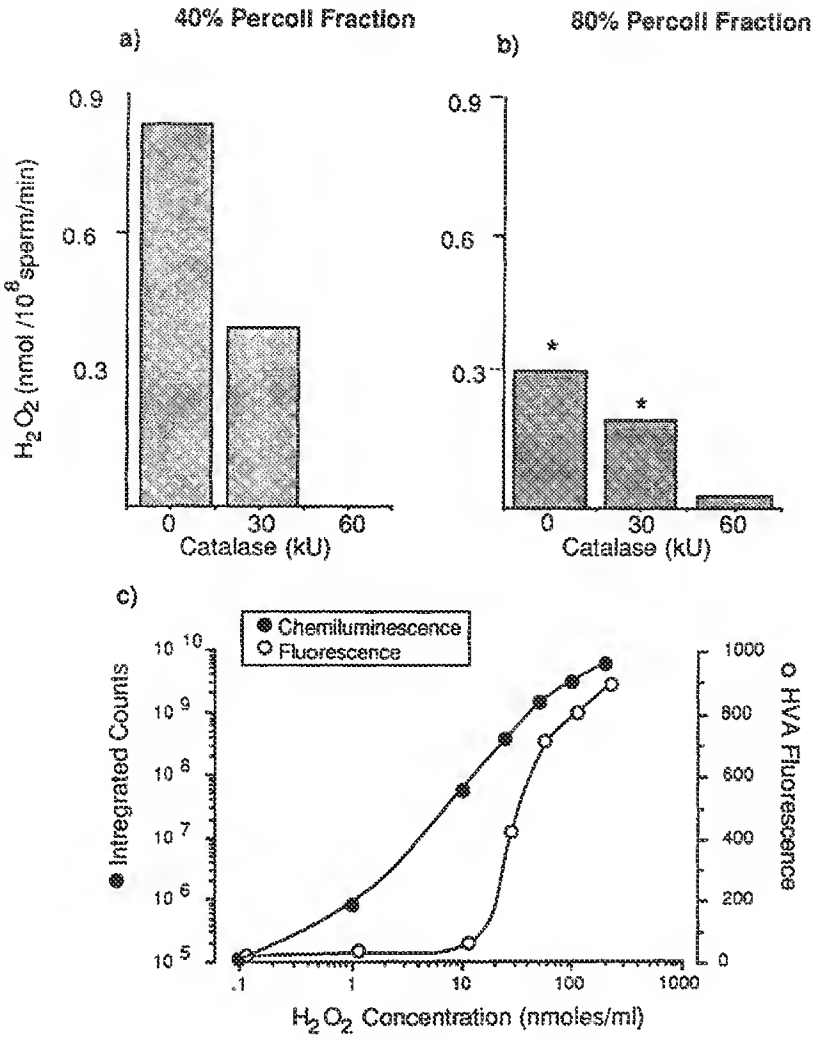

Fig. 4. Measurement of extracellular hydrogen peroxide generation by human spermatozoa using a fluorometric assay based on the HRP-mediated oxidation of homovanillic acid (HVA). Measurements were made on cells isolated on: (a) the low (40%:80% interface) and (b) the high density (base of the 80% fraction) regions of Percoll® gradients and subjected to catalase. Medians are presented; n = 6; * $P < 0.05$ relative to 40% values. (c) Dose response curves for hydrogen peroxide detected by HRP-enhanced chemiluminescence or the HVA-based fluorometric procedure. The standard curve for chemiluminescence extends to concentrations of hydrogen peroxide that are two log orders of magnitude lower than the minimum concentration detected by fluorimetry.

Meizel and Deamer (1978)—it was conceivable that the intracellular peroxidase activity responsible for the luminol-dependent chemiluminescence was located in the acrosome.

Although TMB permitted the detection of peroxidase activity in purified sperm populations by spectrophotometry, the activity per cell was inadequate to permit a direct immunocytochemical localization of this activity. However, a unique opportunity to use a biochemical approach to evaluate the significance of the acrosome as the major source of peroxidase activity in human spermatozoa was presented in the form of a patient exhibiting globozoospermia. This is a rare pathological condition in which the spermatozoa are fully viable and motile but lack an acrosomal vesicle (Syms et al., 1984; Lalonde et al., 1988; Aitken et al., 1990). As revealed in Figure 5c, spermatozoa from this patient possessed a level of peroxidase activity that was considerably reduced relative to the normozoospermic controls. This result suggested that the peroxidase activity expressed by human spermatozoa is concentrated in, but not exclusively confined to, the acrosomal vesicle.

Lucigenin

In contrast to luminol, the chemiluminescent signals obtained with lucigenin were not significantly enhanced by the addition of HRP nor were they suppressed with the peroxidase inhibitors, phenylhydrazine (5 μM) and sodium azide (10 μM) (Fig. 6a,b,e). However, the lucigenin-dependent signals could be suppressed ($P < 0.01$; n = 8) to background levels by doses of SOD ranging from 33 to 875 U/ml, which are comparable with the levels of this enzyme detected in human seminal plasma (149 ± 99 units/ml; Mennella and

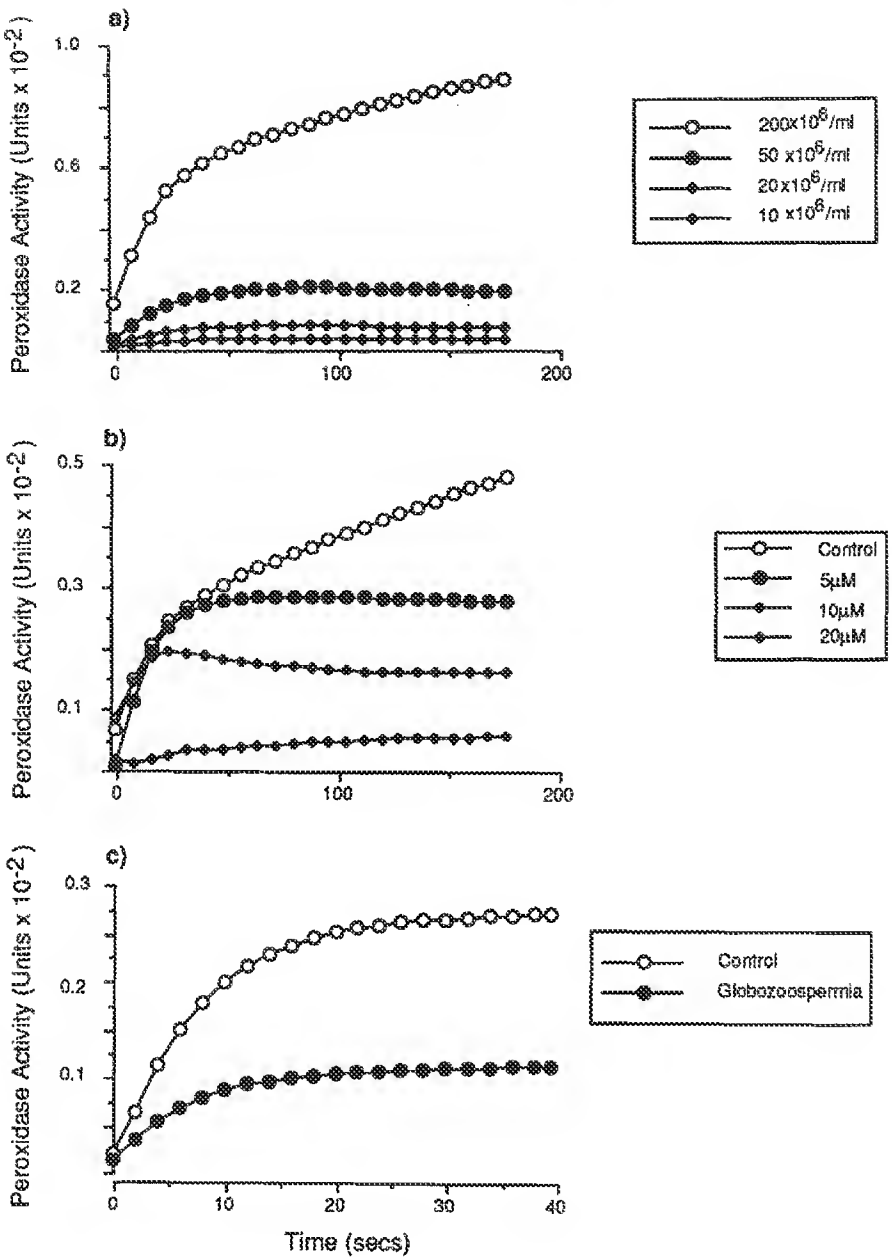

Fig. 5 Peroxidase activity in human spermatozoa monitored using tetramethylbenzidine as substrate (Suzuki et al., 1983): (a) cell concentration dependency, (b) inhibition by phenylhydrazine, (c) reduction in peroxidase activity in a pathological condition, known as globozoospermia, which is associated with the absence of acrosomal vesicles from the spermatozoa.

Jones, 1980). Heat inactivation of the SOD completely destroyed its ability to inhibit the lucigenin-mediated chemiluminescent signal generated by human spermatozoa. Although a statistically significant ($P < 0.01$; $n = 7$) reduction of the lucigenin signal could be obtained with high doses (15–118 kU/ml) of catalase (Fig. 6d) this level of suppression was minor compared with the complete inhibition obtained with SOD (Fig. 6c,d).

In contrast to the KCN-dependent impairment of luminol:HRP mediated chemiluminescence, this inhibitor consistently enhanced both the steady-state, lucigenin-dependent signal and the response of purified human sperm suspensions to agonists such as PMA (Fig. 6f). Although a chemical interaction between KCN and lucigenin generated an enhanced chemiluminescent signal in the absence of spermatozoa (Fig. 6f), this effect was insignificant when compared with the

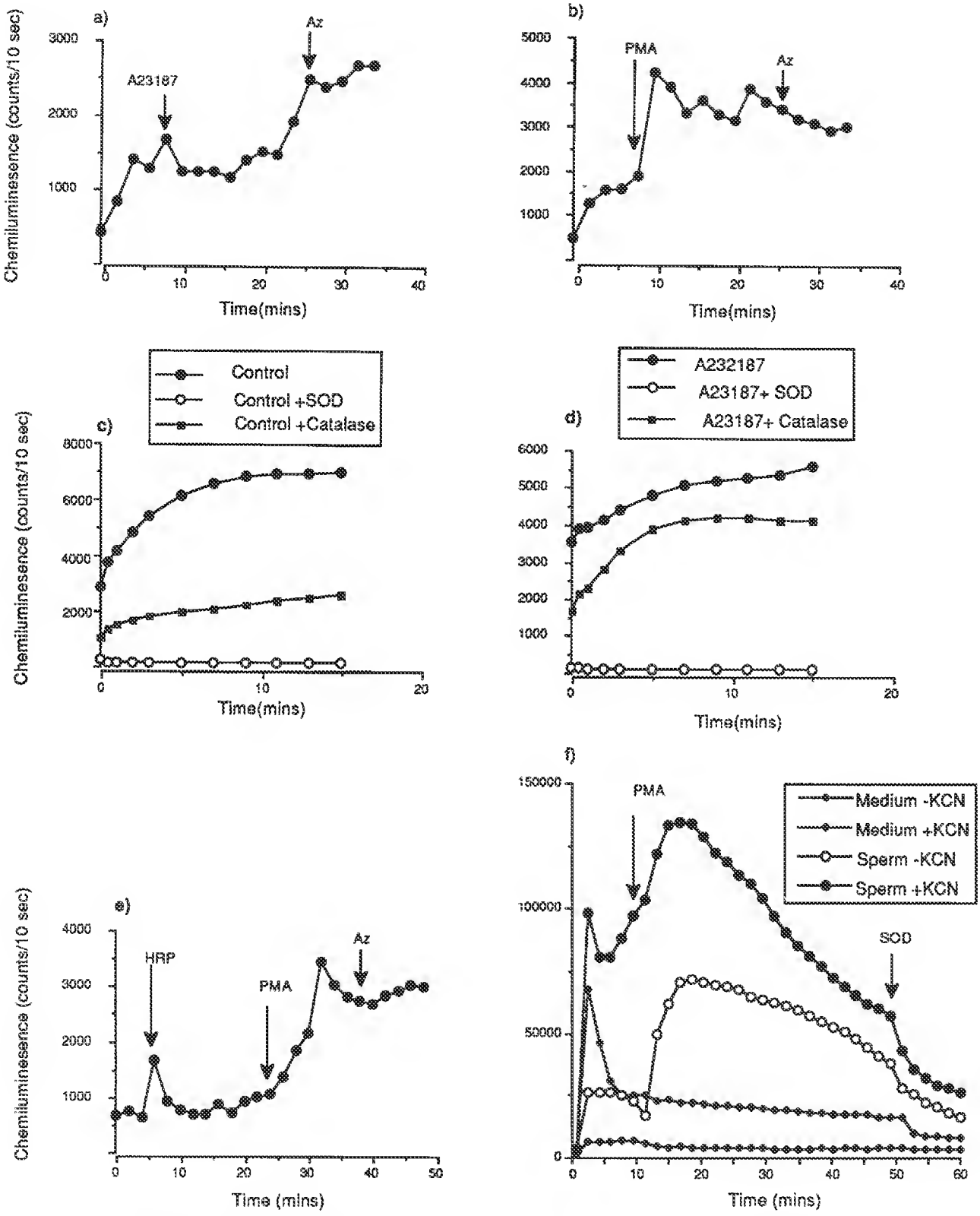

Fig. 6. Lucigenin-mediated chemiluminescence with human spermatozoa. (a,b and e) Failure of 10 μM azide to suppress the lucigenin-dependent signal observed in the presence of (a) 5 μM A23187, (b) 100 nM PMA, and (e) 100 nM PMA + HRP (12 purpurogallin Units/ml). (c and d) 33U SOD completely suppresses the basal and A23187 dependent signals in contrast to the limited inhibition obtained with 23kU catalase. (f) 5mM KCN enhances both the basal and PMA stimulated lucigenin signals.

KCN-enhanced signal given by purified human sperm suspensions. The fact that extracellular SOD could suppress this KCN-induced lucigenin chemiluminescence is consistent with the notion that the changes observed with this reagent are due to the inhibition of intracellular SOD and the resulting enhanced release of superoxide anion into the extracellular space (Fig. 6f).

DISCUSSION

The generation of reactive oxygen species was once thought to be a specialized activity confined to phagocytic granulocytes and macrophages, solely designed to effect the destruction of invading cells and organisms. However, recent research has revealed that oxygen-radical generating systems are present in a wide variety of cell types, including endothelial cells (Sundqvist, 1991) adipocytes (Koshio et al., 1988) germ cells and embryos (Aitken and Clarkson, 1987; Aitken et al., 1989; Nasr-Esfahani et al., 1990) where they are implicated in the etiology of disparate pathological conditions, as well as such physiological processes as signal transduction and second messenger generation (Koshio et al., 1988). In view of the physiological and diagnostic significance of reactive oxygen species generation, the development of sensitive techniques to explore the biochemical basis of this activity is of some importance (Sundqvist, 1991).

The classical technique for measuring superoxide, involving ferricytochrome *c* reduction, is of limited value in the context of human spermatozoa by virtue of its integrative nature, its inability to detect intracellular events, and its lack of sensitivity, requiring sperm concentrations in the order of 0.5×10^8 (Alvarez et al., 1987). The latter is a particular problem in common clinical conditions such as oligozoospermia, where less than 20×10^6 spermatozoa are frequently available for analysis. Similarly, although a number of fluorescence-based techniques are available for measuring hydrogen peroxide generation (Wyman et al., 1987a,b), lack of sensitivity and potential artifacts arising from Raman scattering of the exciting light and cell autofluorescence are limitations inherent in such methods. By contrast, chemiluminescence has been repeatedly shown to be a more sensitive technique than methods based on fluorometric or spectrophotometric end points (Wyman et al., 1987a,b), combining low noise and excellent time resolution with a capacity to detect the low levels of reactive oxygen species production, that are characteristic of human spermatozoa (Aitken and Clarkson, 1987). The interpretation of such data is, however, complicated by the fact that a variety of biochemical pathways can lead to the generation of chemiluminescent signals. The present study was therefore undertaken to establish the cellular mechanisms responsible for the chemiluminescent reactions given by human spermatozoa.

In terms of the luminol-dependent chemiluminescence, existing studies indicate that the chemiluminescent signals generated by these cells is not inhibited by either singlet oxygen or hydroxyl radical scavengers (Aitken and Clarkson, 1987). The results obtained in the present study are consistent with the proposal that the steady-state signal associated with human spermatozoa involves the intracellular oxidation of luminol by oxidants such as hydrogen peroxide or hypochlorous acid, involving the mediation of a sperm peroxidase concentrated, though not exclusively located, within the acrosomal vesicle of the sperm head. Support for the above model can be found in the following observations: (1) the human spermatozoon exhibits maximal peroxidase activity at pH 5.0, equivalent to the pH prevailing in the interior of the acrosomal vesicle; (2) peroxidase activity is severely curtailed in a clinical condition, globozoospermia, in which the spermatozoa are characterized by the lack of an acrosomal vesicle; (3) the spermatozoa of globozoospermic patients do not generate chemiluminescent signals with luminol (Aitken et al., 1990); (4) the basal luminol-dependent signal is relatively resistant to suppression with extracellular SOD and catalase (Aitken et al., 1989); (5) the addition of peroxidase inhibitors azide and phenylhydrazine completely suppresses luminol-dependent chemiluminescence; and (6) this suppressive effect of peroxidase inhibitors is reversed by the addition of an azide-insensitive peroxidase, HRP.

The addition of exogenous HRP enhanced the chemiluminescent signal two- to threefold, presumably by facilitating the dioxygenation of luminol in the extracellular space, by hydrogen peroxide released from the spermatozoa. The sensitivity of HRP-enhanced, luminol-dependent chemiluminescence for extracellular hydrogen peroxide is indicated by the susceptibility of this system to inhibition by catalase. The fact that such signals were also inhibited by SOD may reflect the key role that superoxide is thought to play in the cascade of events responsible for light generation in peroxidase-enhanced chemiluminescent reactions (Vilim and Wilhelm, 1989), during which superoxide anion is generated by the interaction between the luminol radical and ground state oxygen (Hodgson and Fridovich, 1973; Lind et al., 1983; Thorpe and Kricka, 1986). Because superoxide is generated secondarily during the chemiluminescence cascade initiated by hydrogen peroxide, the inhibitory effects of SOD cannot be taken as evidence that $O^{\cdot -}_2$ is a major secretory product of the human spermatozoon (Vilim and Wilhelm, 1989).

The inhibitory effect of azide on the chemiluminescent reactions recorded in this study should be interpreted with care, for although azide is a powerful inhibitor of heme peroxidases, it is also capable of inhibiting other heme - containing enzymes. Human spermatozoa, e.g., are thought to contain small amounts of catalase (Jeulin et al., 1989), which is sensitive to azide inhibition, although in this specific case, azide would be expected to increase luminol-dependent chemiluminescence, not decrease it, as indicated in Figure 1a,b. More significant would be the ability of azide to inhibit any cytochromes, analogous to the cytochrome b_{558} of neutrophils, in the electron transport chain responsible for reactive oxygen species generation in the sperm plasma membrane. However, if this were the case, we should not expect the suppressive effects of azide to be reversed by the addition of exogenous HRP. Thus the most likely explanation for the suppressive effects of azide on sperm chemiluminescence is the inhibition of endogenous heme peroxidases in the same way that this inhibitor is thought to suppress the luminol-dependent chemiluminescence of phagocytes through the inhibition of myeloperoxidase (Peterhans et al., 1988).

Similar caution should be used in the interpretation of experiments involving KCN, which was primarily employed as an inhibitor of Cu/Zn SOD (Alvarez et al., 1987) but which will also inhibit a variety of heme-containing enzymes. The ability of KCN to reduce luminol-dependent chemiluminescence in the presence of HRP contrasts with the inhibitory effects of azide, which are reversed by the addition of this peroxidase. It therefore appears unlikely that the suppressive effects of KCN are mediated by the inhibition of endogenous heme peroxidases. Similarly, although KCN is a recognized inhibitor of catalase, such an action would be expected to stimulate HRP-enhanced chemiluminescence, and yet Figure 3d clearly reveals a suppression of this activity. Furthermore, Figure 3d reveals that exogenous catalase is fully active when added in the presence of KCN, suggesting that, under the experimental conditions employed, the concentration of this reagent is insufficient to achieve an effective suppression of catalase activity. The inhibition of cytochromes in the sperm plasma membrane is another intriguing possibility, although this would not be consistent with the simultaneous enhancement of superoxide generation, as indicated by the concomitant rise in lucigenin-dependent chemiluminescence. Hence, although a variety of alternative mechanisms may be operative, a rational interpretation of these data is that KCN is exerting its effects on human spermatozoa through the inhibition of endogenous Cu/Zn superoxide dismutase, as suggested by Alvarez et al. (1987). As a consequence of this action, hydrogen peroxide release is inhibited and the HRP-enhanced, luminol-dependent chemiluminescence declines. Conversely, superoxide secretion by the spermatozoa is increased, leading to a rise in lucigenin-dependent chemiluminescence. Evidence in support of lucigenin's ability to detect superoxide release has been presented in previous studies (Gyllenhammer, 1987) and is supported by: (1) the inability of peroxidase inhibitors or HRP to influence the lucigenin signal, and (2) the sensitivity of this form of chemiluminescence to suppression with SOD as opposed to catalase.

The above model for luminol-dependent chemiluminescence in human spermatozoa is similar in principle to the scheme proposed for neutrophils in which this probe is held to undergo an intracellular oxidation under the influence of hydrogen peroxide and myeloperoxidase (Dahlgren and Stendahl, 1983; De Chatelet et al. 1982; Vilim and Wilhelm, 1989). The sensitivity of this system to hydrogen peroxide generation is important, since in vitro experiments involving the xanthine:xanthine oxidase oxidant-generating system indicate that it is hydrogen peroxide, rather than superoxide, which is cytotoxic to human spermatozoa (R.J. Aitken, unpublished observations). Furthermore, the enhanced chemiluminescent signals generated in the presence of exogenous HRP are of value in the design of diagnostic systems for monitoring the reactive oxygen species produced by human spermatozoa. This is an important issue because spermatozoa exhibit weak chemiluminescent signals, which need to be amplified as much as possible, if they are to be detected readily.

In conclusion, this study has shed light on the cellular mechanisms responsible for the chemiluminescent signals generated by human spermatozoa and provided a rationale for the design of more refined protocols to differentiate between the intra- and extra-cellular generation of superoxide anion and hydrogen peroxide by these cells. Such protocols should have immediate impact on the diagnosis of sperm dysfunction and also aid in the design of experiments to elucidate the physiological significance of reactive oxygen species generated by human spermatozoa.

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EXHIBIT B

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Development of a novel electrophoretic system for the isolation of human spermatozoa

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BACKGROUND: Optimization of assisted conception outcomes involves the development of rapid, safe, effective techniques for the isolation of functional human spermatozoa free from significant DNA damage. In this study we describe a novel electrophoretic sperm isolation technique that achieves these objectives. METHODS: The separation system consisted of a cassette comprising two 400 μl chambers separated by a polycarbonate filter containing 5 μmol/l pores and bounded by a 15 kDa polyacrylamide membrane to allow the free circulation of buffer. Semen was introduced into one chamber, current applied (75 mA at variable voltage) and within seconds a purified suspension of spermatozoa could be collected from the adjacent chamber. These cells were assessed for their count, viability, motility, morphology and DNA integrity. RESULTS: The suspensions generated by the electrophoretic separation technique contained motile, viable, morphologically normal spermatozoa and exhibited low levels of DNA damage. Moreover, these cell suspensions were free from contaminating cells, including leukocytes. The technique was comparable to discontinuous gradient centrifugation except that it took a fraction of the time and generated cells with significantly less DNA damage. CONCLUSION: Electrophoretic separation represents a highly effective, novel approach for the isolation of spermatozoa for assisted conception purposes.

Key words: DNA damage/electrophoretic method/human spermatozoa/morphology/sperm isolation

Introduction

Assisted conception technologies require the development of techniques for the isolation of human spermatozoa free from cellular contamination and iatrogenic damage (Aitken and Clarkson, 1988; Mortimer, 1991). Human semen samples are intricate cellular mixtures comprising precursor germ cells, subpopulations of viable and moribund spermatozoa, variable amounts of debris and multiple leukocyte subtypes, all suspended in a complex biological fluid, seminal plasma. Any new sperm separation technology has to achieve the rapid physical isolation of viable, functional spermatozoa from human semen, in a manner that optimizes sperm recovery rates, minimizes trauma and prevents oxidative stress (Aitken and Clarkson, 1988). Spermatozoa are very vulnerable to oxidative stress by virtue of their high cellular content of unsaturated fatty acids and limited protection by cytoplasmic antioxidant enzymes (Aitken and Clarkson, 1987; Aitken and Fisher, 1994; Saleh and Agarwal, 2002). Moreover, most human ejaculates contain leukocytes that are in an activated state and generate copious quantities of reactive oxygen species (ROS) (Aitken and West, 1990; Aitken and Baker, 1995; Aitken *et al.*, 1995). Seminal plasma compensates for this intrinsic lack of antioxidant protection by being an extremely rich source of ROS metabolizing enzymes and small molecular mass, free radical scavengers such as vitamin C or uric acid (Lewis *et al.*, 1995; 1997; Potts *et al.*, 2000; Rhemrev *et al.*, 2000). As soon as seminal plasma is removed, the spermatozoa become vulnerable to free radical attack by contaminating leukocytes and both sperm function and DNA integrity can be compromised (Aitken and Clarkson, 1988; Twigg *et al.*, 1998b). It is for this reason that most sperm separation strategies in current practice (swim-up, swim-down or gradient density centrifugation) isolate spermatozoa directly from whole semen without prior removal of the seminal plasma (Aitken and Clarkson, 1988). This procedure has to be performed rapidly because human seminal plasma becomes cytotoxic post-ejaculation largely due to a rapid rise in osmolarity (Aitken *et al.*, 1996). It is also important that sperm isolation procedures involve the minimum of physical trauma because the shearing forces associated with centrifugation stimulate ROS generation in human semen samples (Aitken and Clarkson, 1988; Shekarriz *et al.*, 1995).

While techniques such as swim-up and density gradient centrifugation perform adequately with normal semen samples containing large subpopulations of vigorously motile spermatozoa, these techniques are time consuming and do not avoid the damaging effects of centrifugation. In light of these considerations, we have evaluated the feasibility of a novel

approach to sperm isolation, based on the electrophoretic separation of these cells on the basis of their size and charge.

Materials and methods

All reagents except those otherwise indicated where obtained from Sigma (St Louis, MO, USA).

Semen samples

Human semen samples were obtained from 31 healthy donors of unknown fertility status after at least 48 h abstinence. Samples were allowed to liquefy at room temperature and were then subjected to a routine semen analysis using the guidelines set out by the World Health Organization (1999). Results of these analyses indicated that the majority of samples were in the high normospermic range (sperm concentration 52 ± 5.2 × 10^6; vitality 83 ± 1.5%; motility 72 ± 2.1%). All procedures were approved by the University of Newcastle Human Ethics Committee.

Electrophoretic sperm isolation

The electrophoresis-based Microflow® technology for the separation of spermatozoa by size and charge consists of four separate compartments: two inner chambers (inoculation and collection) and two outer chambers. The outer chambers were separated from the inner chambers by two polyacrylamide restriction membranes with a pore size of 15 kDa (Life Therapeutics, Sydney, Australia), which allowed the movement of small molecules, water and ions between the inner and outer chambers and yet retained the cell suspension within the inner chamber. The outer chambers housed the platinum-coated titanium mesh electrodes and two 12 V buffer pumps (one for each electrode chamber) running at 5 V, to circulate buffer through the chambers at a flow rate of 1.6 l/min. The two inner compartments comprised a 400 µl inoculation chamber into which semen is deposited and a 400 µl collection chamber containing buffer. These two chambers were separated by a polycarbonate separation membrane (5 µmol/l) with an active membrane area of 30 × 15 mm (Figure 1A). The pore size was chosen to permit the transit of spermatozoa across the polycarbonate membrane while excluding larger cells (leukocytes and/or precursor germ cells) that commonly contaminate the human ejaculate.

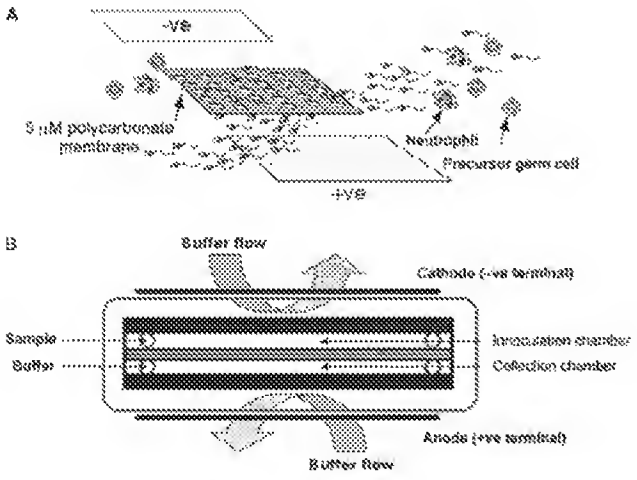

Figure 1. Schematic representations of the cartridge-based electrophoretic separation technology used for this study. (**A**) Directional movement of competent spermatozoa in the applied electric field and size-exclusion of contaminating cell populations facilitated using polycarbonate 5 µm separation membranes. (**B**) Topography of the cartridge configuration including restriction and separation membranes, buffer flows and sample inoculation and collection locations.

Sample solution and buffer were loaded into the two reservoirs (Figure 1B) and allowed to equilibrate for 5 min prior to application of an electric field. The separation and electrode buffer employed in these studies comprised 10 mmol/l HEPES (ICN Biomedicals Inc., Aurora, OH, USA), 30 mmol/l NaCl and 0.2 mol/l sucrose; the pH was adjusted to 7.4 while the osmolarity was 310 mOsm/l. The samples were run at 23°C with a constant applied current of 75 mA and a variable voltage of between 18–21 V. A schematic of the operation of the Microflow® is shown in Figure 1A and B.

Percoll gradient centrifugation and repeated Biggers–Whitten–Whittingham centrifugation

Percoll® fractionation was performed on a discontinuous two-step Percoll gradient. For this procedure, an isotonic solution was prepared by adding 90 ml of Percoll (Pharmacia LKB, Uppsala, Sweden) to 10 ml of 10 × Ham's F10 (ICN Biochemicals) supplemented with 100 mg of polyvinyl alcohol (PVA), 3 mg of sodium pyruvate, 0.37 ml of a 60% sodium lactate syrup and 200 mg of sodium hydrogen carbonate to give an isotonic preparation that was designated 100% Percoll (Lessley and Garner, 1983). This solution was diluted 1:1 with HEPES-buffered (20 mmol/l) Biggers–Whitten–Whittingham (BWW) medium (Biggers *et al.*, 1971), supplemented with 1 mg/ml PVA and discontinuous gradients were created by layering 3 ml of this low-density Percoll preparation above 3 ml of isotonic Percoll. Liquefied semen was layered onto the gradient and centrifuged for 20 min at 500*g*. Spermatozoa were then recovered from the base of the gradient and, for the chemiluminescence experiments, the low-density/high-density Percoll interface. Sperm counts were then performed and cells resuspended to a final concentration of 5 × 10^6/ml for further analysis. Similarly, for the repeated centrifugation protocol, samples were centrifuged for 5 min at 500*g*, following dilution of the semen 1:1 with HEPES-buffered (20 mmol/l) BWW medium supplemented with 1 mg/ml PVA. The pellet was subsequently resuspended in 6 ml of BWW and the centrifugation–resuspension cycle repeated a further two times. Sperm counts were finally performed and, unless otherwise indicated, the spermatozoa resuspended to a final concentration of 5 × 10^6/ml for further analysis.

Sperm and round cell number

Cell counts were conducted using an approved Neubauer haemocytometer following dilution with a formalin/bicarbonate diluent, as specified by the World Health Organization (1999).

Vitality

The vitality of sperm suspensions was assessed using the eosin dye exclusion test. Ten millilitres of 0.05% eosin dye solution in phosphate-buffered saline (PBS) was mixed with 10 ml of sample and examined by phase-contrast microscopy at 400× magnification. A minimum of 200 cells was scored for each sample and results recorded as percentage of live cells.

Sperm motility

Spermatozoa were wet-mounted on slides pre-warmed at 37°C and assessed for the percentage of motile cells using phase-contrast microscopy. For this purpose, at least 200 cells were scored immediately after preparation at 400× magnification.

Computer-assisted sperm assessment

A 10 ml sample of spermatozoa was aliquoted onto a pre-warmed disposable slide with a fixed chamber depth 30 mm. Motion parameters were then captured using a 240 V B/W CCD Camera (Panasonic, Belrose, NSW, Australia) at a frame rate of 50 Hz and recorded using dark-field illumination on professional-grade Super VHS videotape with a Super VHS videotape recorder (JVC, Yokohama, Japan). Samples were evaluated using the HTM-IVOS (Hamilton-Thorn Corporation, Danvers, MA, USA) computer-aided sperm analyser (CASA). The parameter settings applied were as follows: frames = 30 at 50 Hz; minimum contrast = 10; minimum size = 3; non-motile head size = 5; non-motile intensity = 90; threshold average path velocity value = 25 μm/s; slow cells motile = no. A minimum of 100 cells was analysed per treatment population.

Morphology

Samples were smeared onto pre-prepared poly-L-lysine coated slides and allowed to dry at room temperature. Dried smears were fixed in 95% ethanol (Fronine, Riverstone, NSW, Australia) for 15 min then stained by a modification of the Papanicolaou method, as described by the World Health Organization (1999). Smears were re-hydrated for 3 min in 50% ethanol, rinsed for 10 s in dH_2O and stained with Harris' haematoxylin (Fronine) for 3 min. Smears were then washed twice with running tap H_2O for 5 min, separated by an acid ethanol (0.25% HCl in 70% ethanol) treatment for 2 s. Following a brief 1 s dip in dH_2O, smears were progressively dehydrated in 50, 70, 80 and 95% ethanol for 10 s then incubated with Orange G6 (Fluka, Buchs SG, Switzerland) cytoplasmic stain for 2 min. Surplus stain was removed by 95% ethanol for 20 s and EA-50 cytoplasmic and nucleolar staining performed for 5 min. Smears were then dehydrated (95% ethanol for 15 s followed by 100% ethanol for 2 min), allowed to completely air-dry and mounted using DPX media (BDH laboratory supplies, Poole, UK). Morphological examination was performed using a Zeiss Axioplan 2 (Ziess, Oberkochen, Germany) microscope using a 100× oil-immersion objective and a total magnification of 1250×.

The classification and evaluation of sperm morphology was established according to Menkveld *et al.* (1990). The entire spermatozoon (head, neck, midpiece and tail) was taken into consideration for evaluation along with any germinal epithelium or other cell types present. Spermatozoa were classified into one of seven groups, normal (whole sperm), large, small, elongated, duplicated and amorphous heads, all with or without the presence of a 'cytoplasmic droplet' and/or tail, neck and/or midpiece defect (Menkveld *et al.*, 1990). The seventh group consisted of spermatozoa with a normal head with a tail and/or a neck and/or a midpiece defect and/or the presence of a cytoplasmic droplet (Menkveld *et al.*, 1990). Tail, neck and midpiece defects, loose heads, germinal epithelium and unknown cells were recorded separately and expressed per 100 spermatozoa (Menkveld *et al.*, 1990). In addition, the morphological normality of the sperm preparations was assessed using the sperm deformity index (SDI) as described by Panidis *et al.* (1998). For each analysis, a minimum of 100 spermatozoa was analysed with the aid of an eyepiece micrometer.

Chemiluminescence assessment of leukocyte contamination

Semen samples were subjected to a 5 min electrophoretic separation as described previously. At the end of the separation period the separated sperm suspension and the residual semen samples were washed twice in BWW (500*g* for 5 min) prior to chemiluminescence analysis. This wash step was necessary to remove any traces of the antioxidants present in seminal plasma that would have artificially suppressed the chemiluminescent signals generated by the residual sperm population. In order to control for the centrifugation process itself, the separated sperm populations were also centrifuged under identical conditions so that the signals generated by the separated and residual sperm populations could be directly compared. Luminol-dependent chemiluminescence was recorded on a Berthold 953 luminometer (Berthold Detection Systems GmbH, Crown Scientific Pty Ltd, Moorebank, Australia) at 37°C using 400 μl aliquots of spermatozoa at a concentration of 2×10^6/ml. Cell-free medium and dimethyl sulphoxide (DMSO) vehicle controls were recorded with every treatment replicate to ensure sperm suspension-dependent responses. Luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) (10 mmol/l) was prepared in DMSO and diluted with sperm suspensions to give a final concentration of 100 μmol/l. Luminol was supplemented with horseradish peroxidase, freshly prepared as a 2 mg/ml stock solution in electrophoresis buffer, 8 μl of which was added to 400 μl of sperm suspension to give a final peroxidase activity of 11.52 U/ml. Any contaminating leukocytes were activated after 5 min incubation by addition of 20 μl zymosan opsonized with autologous serum and results were recorded as continuous traces and as integrated photon counts over a fixed time period of 20 min. This method gives a linear relationship between chemiluminescence and the concentration of CD45 positive leukocytes over the concentration range (10^3–10^6 leukocytes/ml) typically encountered in human semen samples (see Figure 3C).

DNA damage: TUNEL assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was used to evaluate DNA integrity and was performed using a commercial kit (*In situ* cell death detection kit, Fluorescein; Roche, Mannheim, Germany) as outlined by the manufactures protocol for smears, with the following exceptions. Undiluted labeling solution, in our experience, created high levels of background and non-specific staining which hampered the analysis of TUNEL-positive cells. The labeling solution was therefore diluted 1:5 with filtered PBS. Fractionated sperm cells were washed twice in filtered PBS (1000*g* for 5 min) and cell suspensions fixed in 4% (w/v) paraformaldehyde (ProSciTech, Thuringowa Central; QLD) in PBS at 4°C for in excess of 10 min. Fixed spermatozoa were washed twice and re-suspended in filtered PBS; 10 μl of sperm suspension was then smeared onto poly-L-lysine-coated diagnostic slides and air-dried. Dried smears were permeabilized for 2 min at 4°C using 0.1% Triton X-100 in 0.1% sodium citrate and digested using 100 mg/ml Proteinase K (Promega, Madison, WI, USA) for 15 min at 37°C. Following PBS washing, positive controls were treated with 1 mg/ml DNAse (type 1; Roche) in PBS for 10 min at 37°C, while all other wells were overlaid with 1 × TE buffer. Smears were washed and blocked with 1% BSA (Research Organics, Cleveland, OH, USA) in PBS for 15 min, prior to the application of TUNEL reaction

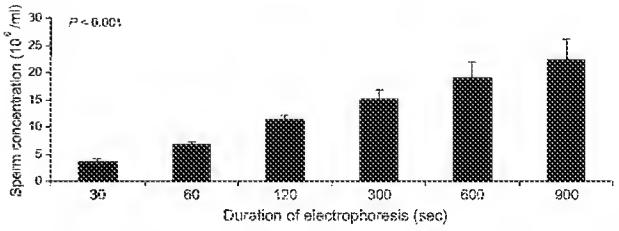

Figure 2. Time-dependent isolation of spermatozoa from unprocessed semen. Tens of millions of spermatozoa were isolated with this system within 5 min ($n = 6$).

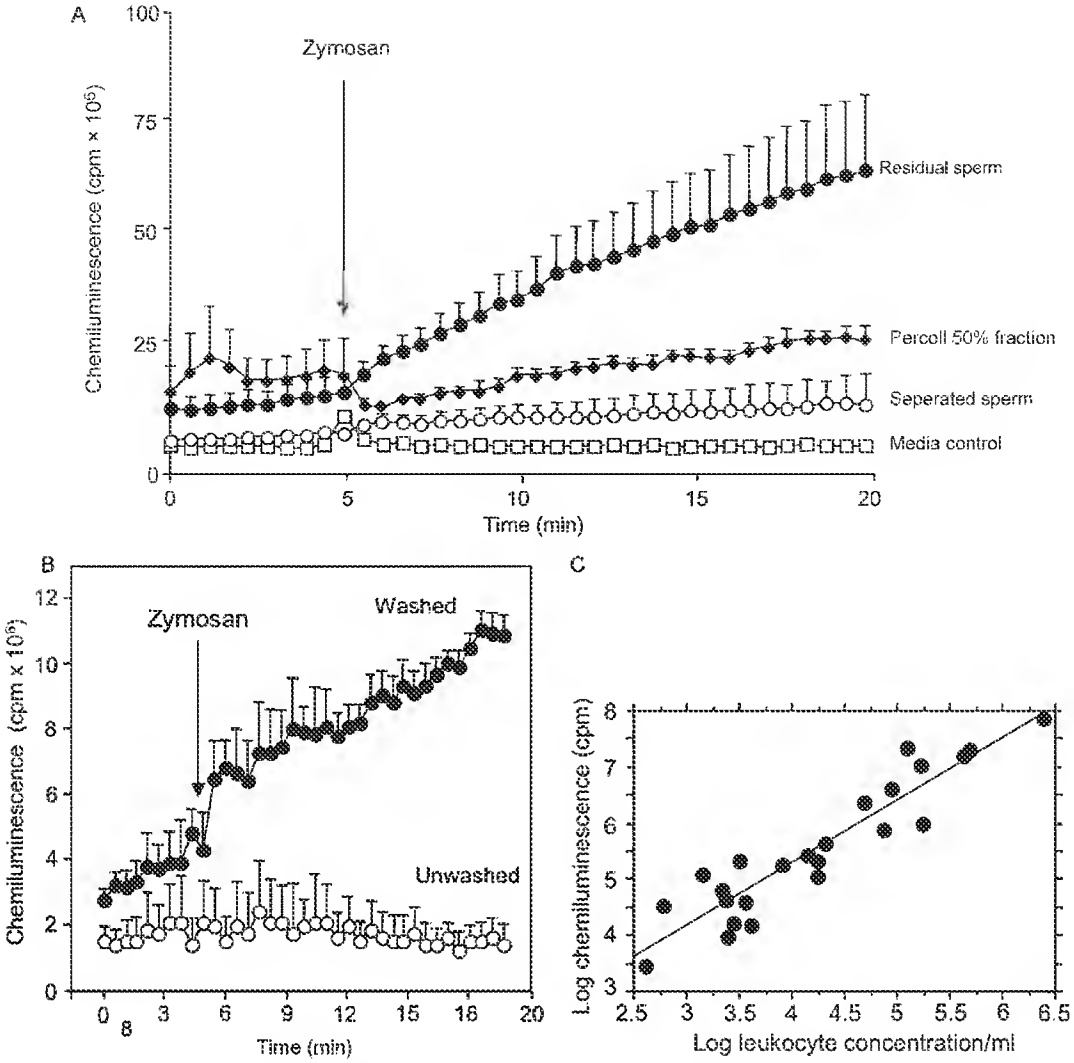

Figure 3. Zymosan elicited chemiluminescence responses generated by populations of spermatozoa in the presence of luminol and horseradish peroxidase. (**A**) Responses generated by washed populations of spermatozoa that had been electrophoretically separated, the cells remaining in the residual semen sample, and cells recovered from the 50%:100% Percoll interface, showing the lack of activity characteristic of the separated sperm populations (**B**) Impact of centrifugation on chemiluminescence responses generated by electrophoretically separated sperm populations to zymosan treatment ($n = 9$). (**C**) Dose-response analysis for the relationship between zymosan-induced chemiluminescence and the concentration of CD45-positive leukocytes.

components. A negative control consisting of nucleotide solution without TdT enzyme was included in all experiments. Ten millilitres of TUNEL treatment mix were layered over each sample and the slides incubated in a humidified atmosphere in the dark for 1 h at 37°C. The slides were subsequently rinsed three times in PBS, mounted in Mowiol (2.4 g Mowiol; Calbiochem, La Jolla, CA, USA), 6 g glycerol, 6 ml dH_20, 12 ml 0.2 mol/l Tris (pH8.5), 2.5% 1,4-diazo-bicyclo-{2.2.2}-octane (DABCO), and viewed using a Zeiss Axioplan2 fluorescence microscope with selective filters for FITC fluorescence. Cells exhibiting fluorescence were scored and results expressed as the percentage of TUNEL-positive cells; a minimum of 100 cells were considered for each analysis.

Statistical analysis

Each experiment was repeated at least three times on independent samples and statistical analysis was performed using Microsoft Excel® 2000 and SuperANOVA (Abacus Concepts Inc., Berkeley, CA, USA). Angular transformations were carried out for percentages prior to statistical analysis using an angular transformation table, where $p = \sin^2$. Averages were calculated for each experiment, as well as standard errors of the mean (SEM) for $n=1$. Post-hoc testing was performed using Fisher's Protected Least Significant Difference (PLSD) and samples with a P-value of <0.05 were considered significant.

Results

Number and quality of spermatozoa

Unfractionated human semen samples were placed in one compartment of the separation cassette and left for 5 min to equilibrate; at this point current was applied. During the 5 min equilibration period spermatozoa migrated to the collection chamber as a consequence of their inherent motility, to give a starting sperm concentration of $1.67 \pm 0.58 \times 10^6$/ml. Within 30 s of applying current this number increased to

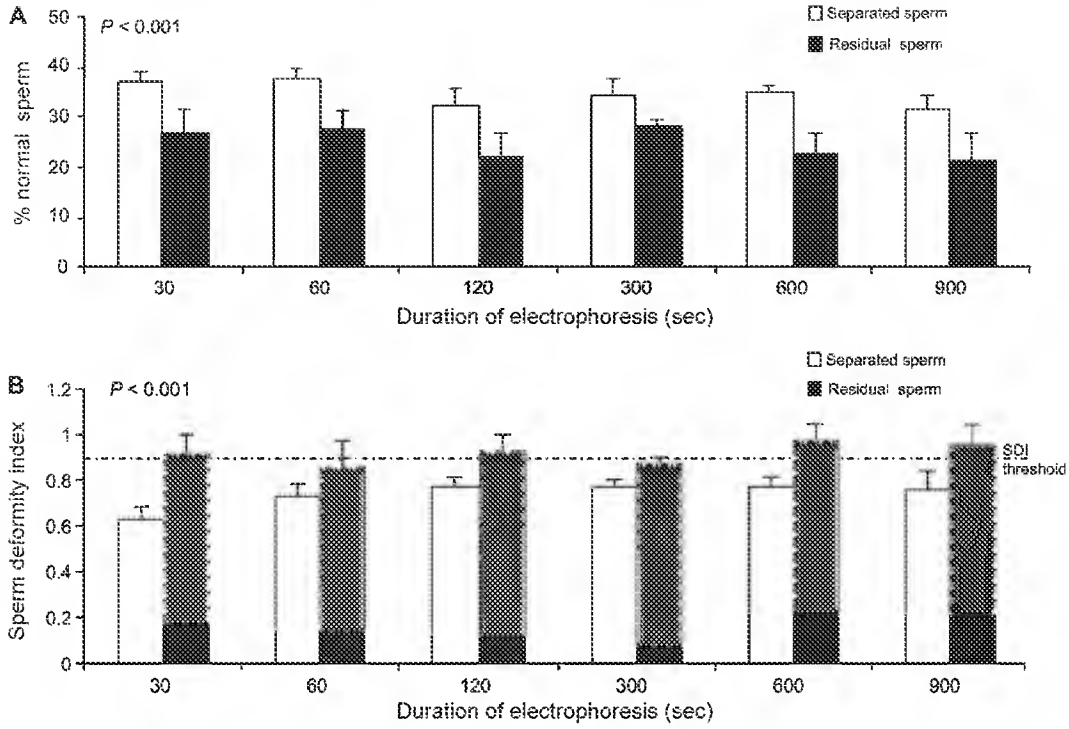

Figure 4. Morphological examination using Papanicolaou staining. (**A**) Percentage number of morphologically normal forms plotted against electrophoresis time. Shown are mean ± SEM ($n = 3$) for residual and separated sperm populations. (**B**) SDI for residual and separated sperm populations plotted against electrophoresis time. SDI values below the indicated threshold of 0.93 (Panidis *et al.*, 1998) are thought to reflect an enhanced capacity for fertilization. Results represent mean ± SEM ($n = 3$).

$3.55 \pm 0.42 \times 10^6$/ml and continued to increase rapidly, thereafter reaching a peak of $22.31 \pm 5.85 \times 10^6$/ml after 900 s (Figure 2). This time-dependent increase in the number of spermatozoa recovered from the collection chamber was highly statistically significant ($P < 0.001$). The importance of the electric field was emphasized in additional experiments in which semen samples were equilibrated for 5 min in the inoculation chamber and then current either was, or was not, applied for another 5 min period. Under these circumstances the yields obtained were $24.6 \pm 6.95 \times 10^6$cells/ml and $4.52 \pm 0.74 \times 10^6$ cells/ml, respectively.

The presence of contaminating round cells was also carefully monitored by phase-contrast microscopy in these electrophoretically separated sperm populations, and found to be undetectable. Moreover, chemiluminescence analysis of potential leukocyte contamination supported the cytological analysis in revealing negligible levels of leukocyte-derived chemiluminescence as compared with the washed residual semen samples and 50% Percoll fractions (Figure 3A). While the act of centrifugation itself generated a weak chemiluminescence signal in the separated population of cells (Aitken and Clarkson, 1988), in the absence of centrifugation, the separated spermatozoa did not generate a chemiluminescent signal in response to zymosan (Figure 3B).

Morphology

An analysis of sperm morphology indicated that the spermatozoa separated using the electrophoretic system possessed a significantly improved morphology compared with the residual excluded population (Figure 4A; $P < 0.001$). This difference was consistent across all time intervals assessed and did not vary significantly with the duration of the electrophoretic treatment. These data were reinforced using an alternative technique for assessing morphology, the sperm deformity index (SDI) introduced by Panidis *et al.* (1998). This assessment technique has been shown to provide a correlation between the morphological status of a given sperm population and its potential for fertilization *in vitro*. In this study, the SDI values recorded for the separated sperm populations was significantly below the threshold SDI value of 0.93 (Panidis *et al.*, 1998) for all electrophoretic time-points (Figure 4B; $P < 0.001$). As SDI values increase above this threshold, the fertilizing capacity of the spermatozoa is held to decrease proportionally (Panidis *et al.*, 1998). The ability of the electrophoretic technique to select a subpopulation of morphologically normal spermatozoa with low SDI values therefore indicates enrichment for spermatozoa with potentially enhanced fertilizing potential.

Viability and motility

The percentage of viable spermatozoa isolated using the electrophoretic separation procedure was consistent with the values recorded for the original ejaculates (Figure 5). Moreover, the vitality of the spermatozoa did not change significantly with the duration of the electrophoretic treatment. Similarly, the percentage of motile spermatozoa did not show any enrichment or decline following electrophoretic separation but remained at a level consistent with that observed in

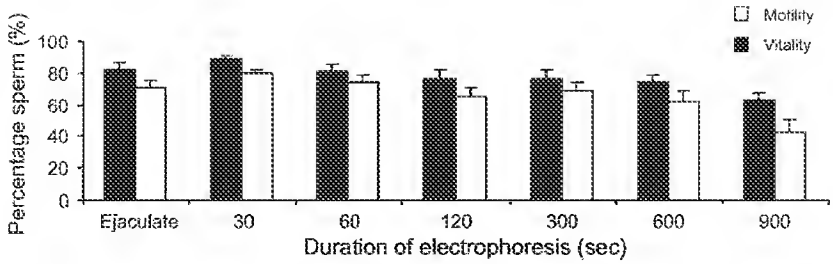

Figure 5. Eosin-exclusion vitality measurements and motility counts for electrophoretically separated sperm populations after 1.5 h incubation in BWW at 37 °C in an atmosphere of 5% CO_2 in air. Corresponding values for the original ejaculates are also shown. Results represent mean ± SEM ($n = 6$).

the ejaculate. Furthermore, the duration of electrophoresis did not change the percentage of motile spermatozoa significantly, although after 900 s a slight reduction in this criterion of semen quality was noted (Figure 5). Analysis of the kinematic characteristics of the spermatozoa by CASA also demonstrated that the quality of sperm motility did not change significantly from the high levels observed in the original semen samples, regardless of the duration of the electrophoretic separation procedure (Figure 6).

DNA integrity

TUNEL analysis revealed that the spermatozoa separated using the electrophoretic system possessed a significantly reduced level of DNA damage compared with the excluded population (Figure 7; $P < 0.05$). This significant difference was observed for all time-points up until 10 min of electrophoresis, after which no further change in the percentage of DNA-damaged spermatozoa was observed.

Comparative analysis of sperm isolation techniques

In order to determine the relative value of the electrophoretic sperm isolation procedure, 400 µl aliquots of individual semen samples were processed using this method, discontinuous Percoll gradients and repeated centrifugation, and the quality of isolated spermatozoa compared (Aitken and Clarkson, 1988).

In terms of sperm recoveries, no significant difference was observed between the average number of spermatozoa isolated by electrophoresis ($11.42 \pm 1.59 \times 10^6$/ml) and Percoll gradient centrifugation ($11.92 \pm 1.42 \times 10^6$/ml). Significantly higher yields were observed using the 3 × centrifugation method (Figure 8A; $P < 0.001$), as might be expected given the non-selective nature of this sperm preparation technique. However, these yields were accompanied by the presence of contaminating round cells ($2.67 \pm 0.60 \times 10^6$/ml) not observed with the aforementioned techniques.

All of the preparative techniques resulted in the preparation of motile, viable spermatozoa that were not significantly different from each other, or from the values recorded for the original ejaculate (Figure 8B). The only exception to this rule was the Percoll gradient technique which resulted in levels of motility that were significantly greater than those observed with any of the other preparative techniques ($P < 0.05$). CASA of average path velocity, straight line velocity and curvilinear velocity was performed to extend these motility data (Figure 8C). CASA revealed that the electrophoretic sperm preparation technique resulted in the isolation of spermatozoa exhibiting movement characteristics that were not significantly different from those prepared on discontinuous Percoll gradients or by repeated centrifugation.

TUNEL analysis revealed that the spermatozoa prepared by electrophoresis and Percoll gradient centrifugation exhibited less DNA damage than those prepared by repeated

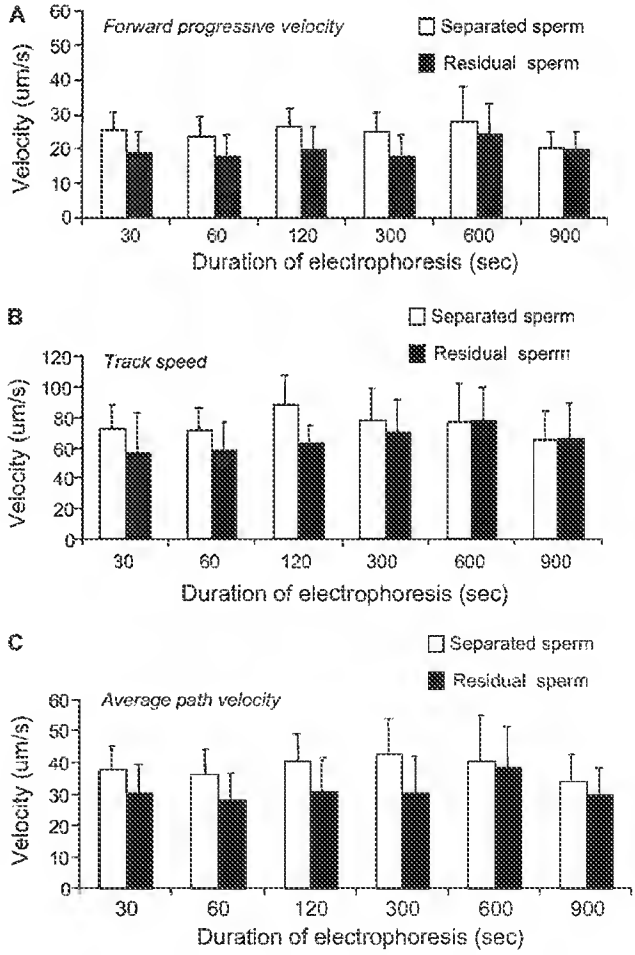

Figure 6. Analysis of the CASA measurements for residual and separated sperm populations recovered after increasing electrophoresis times. (**A**) Forward progressive velocity, (**B**) track speed (curvilinear velocity) and (**C**) average path velocity. Results represent mean ± SEM ($n = 3$).

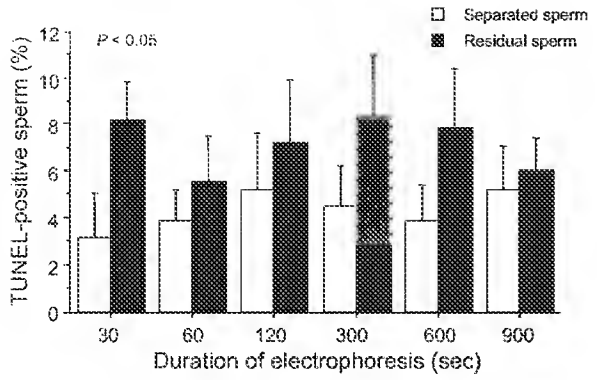

Figure 7. Impact of electrophoretic treatment on DNA integrity measured as percentage of TUNEL-positive cells (mean ± SEM) for residual and separated sperm populations ($n = 6$). The DNase positive control (99.67 ± 0.23%) ensured assay efficacy while the Tdt enzyme negative control (0.0 ± 0.0%) guaranteed that non-specific binding did not obfuscate the analyses.

centrifugation. However, only in the case of the electrophoretically isolated spermatozoa was the level of DNA damage observed significantly lower than that observed in the original ejaculate ($P < 0.05$).

Analysis of the morphological normality of the spermatozoa by ANOVA revealed a significant difference due to treatment; the electrophoretically separated cells exhibiting significantly more normal forms than the other preparative techniques analysed (Figure 9A; $P < 0.05$). Furthermore, analysis of morphology using the sperm deformity index showed a significant reduction in sperm abnormalities for cells separated using the electrophoretic system compared with alternative preparation procedures as well as the original ejaculate (Figure 9B; $P < 0.05$). Particular attention was placed on small-headed spermatozoa that, because of their shape, might have passed across the 5-μm filter more readily than normal cells, despite their lack of morphological normality. However, this analysis revealed no significant difference in the incidence of such cells between electrophoretically separated sperm (14 ± 1.5%), 100% Percoll-separated sperm (15 ± 3.2%) and the original ejaculate (14 ± 2%).

Discussion

Assisted conception techniques are now responsible for 2–4% of new births in developed countries. While this technology has revolutionized the treatment of infertile couples, concerns have been raised about the incidence of birth defects in such children (Hansen *et al.*, 2002). One of the factors thought to be responsible for such morbidity, as well as the high rate of early pregnancy loss associated with assisted conception cycles, is the presence of DNA damage in the fertilizing spermatozoon (Aitken, 2004; Lewis and Aitken, 2004). As a result, it is now imperative that we develop optimized procedures for the isolation and separation of human spermatozoa for assisted conception that maintain DNA integrity (Zini *et al.*, 2000) and minimize ROS production (Aitken and Clarkson, 1987), while maximizing

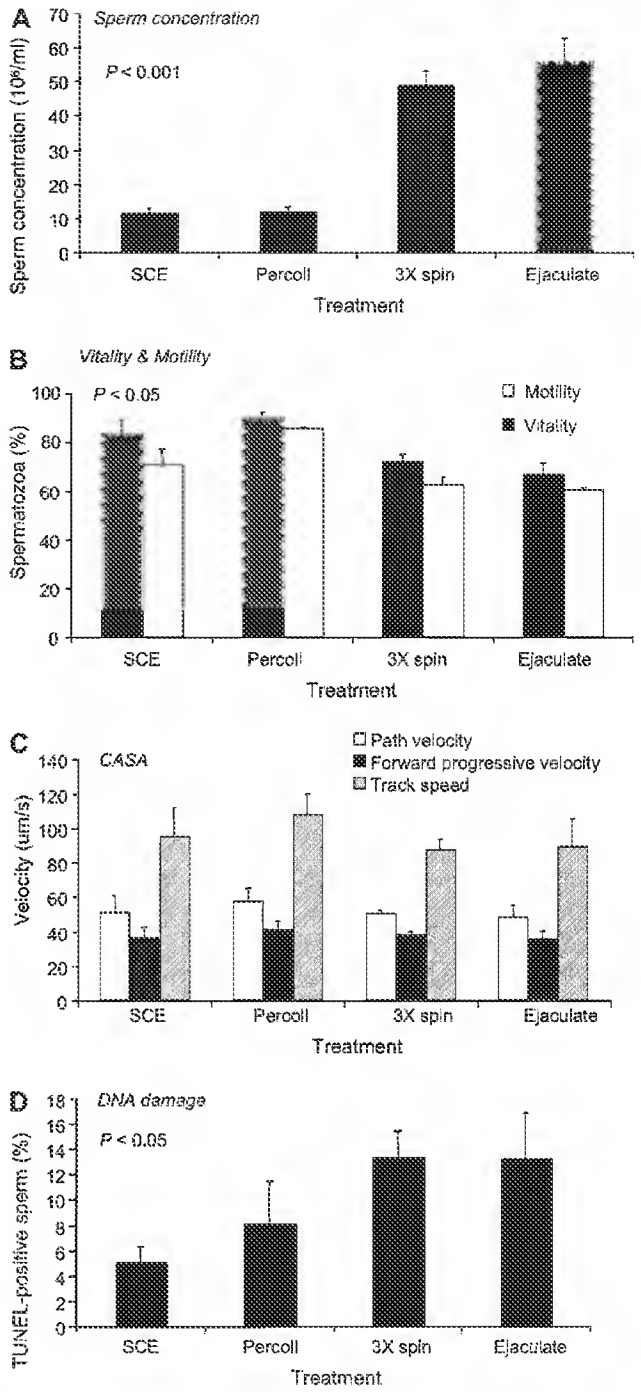

Figure 8. Comparison of sperm suspensions isolated by electrophoresis (SCE = sperm cell electrophoresis), Percoll® gradient centrifugation (Percoll) and repeated centrifugation (3×spin), in relation to the original ejaculate. (**A**) Sperm concentration of preparations represented in millions of cells per ml. Values represent mean ± SEM ($n = 4$). Overall significant difference due to preparation technique by ANOVA ($P < 0.001$). (**B**) Eosin-exclusion vitality measurements and motility counts after 1.5 h incubation in BWW (37 °C; 5% CO_2). Values represent mean ± SEM ($n = 3$). Overall significant difference due to preparation technique by ANOVA ($P < 0.05$). (**C**) CASA parameters. Values represent mean ± SEM ($n = 3$). (**D**) Treatment-mediated DNA damage expressed as percentage TUNEL-positive sperm. Values represent mean ± SEM ($n = 6$). Overall significant difference due to preparation technique by ANOVA ($P < 0.05$).

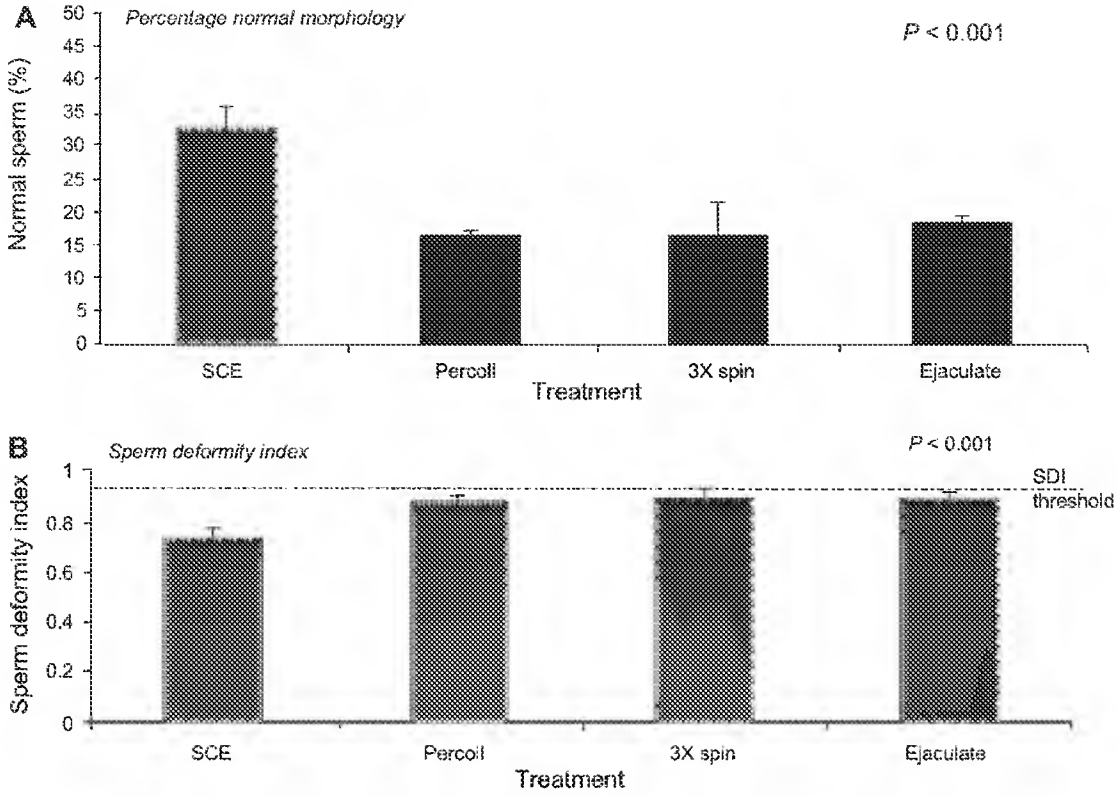

Figure 9. Comparative analysis of the morphology of spermatozoa using Papanicolaou staining. (**A**) Percentage of morphologically normal forms generated with various preparative techniques. Values represent mean ± SEM ($n = 4$). Significant difference due to preparation technique ($P < 0.001$). (**B**) Sperm deformity indices (SDI) for spermatozoa generated using various preparative techniques. SDI values below the indicated threshold of 0.93 (Panidis *et al.*, 1998) are thought to reflect an enhanced capacity for fertilization. Values represent mean ± SEM ($n = 4$). Significant difference due to preparation technique ($P < 0.001$).

functional competence. The need to develop alternative techniques for the effective recovery of high quality spermatozoa prompted this investigation.

Our data indicate that high numbers of viable, motile spermatozoa exhibiting low levels of DNA damage and high levels of morphological normality can be isolated from human semen utilizing a sperm separation strategy based on sperm size and electronegative charge. The importance of cell size as a selection criterion is self-evident, spermatozoa being one of the smallest cells in the body and, importantly, significantly smaller that the major contaminants in human semen, precursor germ cell and leukocytes.

Although a low-level chemiluminescence signal was generated by the electrophoretically separated cells (Figure 3B), this could be attributed to the washing steps that had to be employed prior to chemiluminescence in order to permit direct comparison of the residual and separated sperm populations. The physical shearing forces associated with repeated centrifugation have previously been shown to enhance the chemiluminescent activity of human spermatozoa (Aitken and Clarkson, 1988). In the absence of centrifugation, the electrophoretically separated sperm populations did not generate a chemiluminescence signal in response to zymosan (Figure 3B). Such results emphasize how minimally traumatized these electrophoretically prepared sperm suspensions were (Figure 3B) and the extent to which leukocyte contamination had been avoided.

The importance of cell charge is in keeping with existing data indicating that normal human spermatozoa possess a glycocalyx that is rich in sialic acid residues (Kallajoki *et al.*, 1986; Caldaza *et al.*, 1994). The existence of a positive correlation between the electro-negativity of human spermatozoa and the quality of these cells may be partly related to the presence of CD52, a highly sialated, lipid-anchored molecule that is acquired by spermatozoa during epididymal transit (Kirchhoff and Schroter, 2001). Thus, the negative charge associated with high quality spermatozoa may simply reflect the fact that these cells have differentiated normally in the testes and entered the epididymis in a sufficiently operational state to participate in the massive cell–cell transfer of GPI-anchored CD52 to the sperm surface (Schroter *et al.*, 1999). This molecule is thought to play an important role in the capacitation of human spermatozoa. As sperm capacitate, CD52 moves from a widely distributed surface pattern to become concentrated in the equatorial region of these cells. Male infertility is associated with a significant decrease in the percentage of spermatozoa expressing CD52 and a reduced percentage of cells exhibiting the equatorial pattern of localization following capacitation (Giuliani *et al.*, 2004). Furthermore, this pattern of CD52 expression was found to be highly correlated with sperm morphology (Giuliani *et al.*, 2004), suggesting at least one reason why electrophoretically separated spermatozoa might be characterized by excellent morphology. Preliminary experiments have established that

neuraminidase treatment of human spermatozoa interferes with their isolation using the electrophoretic system (C.Ainsworth, unpublished observations). Such results are in keeping with the proposed importance of sialation in establishing the charge differences that underpin the electrophoretic method of sperm separation. The involvement of CD52 in establishing the overall sialation status of human spermatozoa is currently under investigation.

Whatever biochemical principles underpin the electrophoretic sperm isolation procedure, this method is clearly capable of isolating large numbers of spermatozoa that are viable, motile, morphologically normal and relatively free of DNA damage and contaminating cells. Electrophoretic separation compared favourably with Percoll gradient centrifugation in terms of the purity of the sperm population, the lack of ROS generation, as well as the vitality and morphological normality of the isolated cells. The electrophoretic procedure was also superior to Percoll gradient centrifugation in terms of the time taken to isolate the spermatozoa and the lack of physical trauma. The fact that neither a centrifuge nor a skilled technician are necessary for this sperm isolation procedure also means that it could be readily adopted as an office procedure in order to isolate spermatozoa for assisted conception purposes. In principle, a semen sample would simply have to be inoculated into one chamber of the cassette, current applied for 5 min and the isolated spermatozoa removed, diluted with culture medium and used for therapeutic purposes.

The lack of DNA damage is a particularly valuable feature of the electrophoretic sperm separation technique. DNA damage in the male germline has been associated with impaired fertility following natural conception; the time to pregnancy increasing as a function of the proportion of sperm with abnormal chromatin (Spano *et al.*, 2000; Loft *et al.*, 2003). Furthermore, DNA damage in spermatozoa has been linked with poor conception rates following IVF (Sun *et al.*, 1997; Aitken, 2004). However, this is not consistently the case with ICSI (Lewis and Aitken, 2004). With this technique, successful fertilization can be achieved despite high levels of DNA damage in the injected spermatozoon, regardless of whether ejaculated or testicular spermatozoa are used in the course of therapy (Twigg *et al.*, 1998a; Aitken, 2004; Gandini *et al.*, 2004; Lewis and Aitken, 2004). This might be expected, since with ICSI fertilization does not depend on the functional competence of the spermatozoa in terms of the ability of these cells to capacitate, acrosome react, penetrate the zona pellucida and fuse with the vitelline membrane of the oocyte. However, while ICSI might permit fertilization with DNA-damaged spermatozoa, such success might be achieved at some cost to the embryo. According to recent studies, the post-fertilization development of human embryos can be seriously impaired by the use DNA-damaged spermatozoa in assisted conception (Bungum *et al.*, 2004; Lewis and Aitken, 2004; Virro *et al.*, 2004). Such disruption of embryonic development is presumably associated with the abortive transcription of damaged genes originating from the paternal genome, as well as possible epigenetic effects (Braude *et al.*, 1988; Tesarik *et al.*, 2001). Thus, the ability of the electrophoretic sperm isolation method to select germ cells exhibiting low levels of DNA damage with great rapidity and efficacy should be particularly valuable in the delivery of assisted conception services.

Notwithstanding the ability of the electrophoretic system to isolate spermatozoa enriched for normal morphology and low levels of DNA damage, this procedure did not enrich for sperm motility. This is not an unexpected finding, because previous studies have found that the electrophoresis of spermatozoa is detrimental to their motility (Engelmann *et al.*, 1988). In the comparative wing of this study, the quality of sperm motility, as assessed by CASA, was no different regardless of whether the spermatozoa were prepared by Percoll gradient centrifugation or the electrophoretic method. However, there was a decrease in the percentage of motile spermatozoa with the latter, compared with the Percoll technique. We interpret these findings as indicating that electrophoresis of spermatozoa disrupts motility in a subset of vulnerable spermatozoa, possibly by interfering with the regulation of ion fluxes across the sperm plasma membrane. If only moribund spermatozoa are negatively affected by the electrophoretic procedure then there should be little impact on the fertilizing capacity of the sperm suspension as a whole. Clinical trials are being conducted to address this issue in the context of assisted conception therapy. Of course, such factors are of little concern if ICSI is to be performed. Indeed, one of the exciting prospects for this method of sperm isolation, is its potential application in the isolation of spermatozoa exhibiting low levels of DNA damage from complex cellular mixtures such as testicular or epididymal biopsies. In principle this electrophoretic sperm isolation procedure has great potential as an extremely versatile, time- and cost-effective method for preparing spermatozoa for a wide variety of assisted conception applications. It must be emphasized, however, that this proof-of-principle study has been confined to the analysis of normospermic donors. Additional studies will be required with seriously compromised, pathological semen samples to confirm the effectiveness of this electrophoretic technique in the management of male infertility.

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